d-FRUCTO-PYRANOSE, A SUGAR UNFERMENTABLE BY YEAST

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Slator (1908) showed that living yeast of various species ferments d-fructose at the same rate as d-glucose over a wide range of concentrations. Hopkins (1931) and Hopkins and Roberts (1935a) confirmed the approximate equality of the rates of fermentation of d-fructose and d-glucose in concentrations above 1 p.c. Below this concentration, however, d-fructose is fermented more slowly than d-glucose. Thus, at a concentration of 0·1 p.c. the rate of fermentation of fructose at 30° C. is only 63 p.c. of that of glucose. Furthermore, while the temperature coefficient of fermentation of glucose from 15° C. to 40° C. is independent of the concentration of glucose between 0·25 and 5·0 p.c., for fructose this is true only between 1·0 and 5·0 p.c. Between 1·0 and 5·0 p.c. the temperature coefficients are approximately the same for both sugars (Slator, 1908). For fructose at 0·25 p.c. the temperature coefficient of fermentation is in close agreement with the corresponding temperature coefficient of mutarotation of β-d-fructo-pyranose to equilibrium (Hopkins and Roberts, 1935b). To explain these results Hopkins and Roberts (1935b) have suggested that in a solution of d-fructose at equilibrium one component only, probably fructo-furanose, is fermented and that the rate at which it is replaced from the other components limits the rate of fermentation at low concentration. Hudson (1909), Isbell and Pigman (1938) and others have demonstrated that the mutarotation of β-d-fructo-pyranose, the only modification of d-fructose known in the crystalline state, is mainly a pyranose-furanose interconversion.

Hopkins' ingenious theory has as yet received little attention. Obviously direct experimental evidence that d-fructo-pyranose is unfermentable by yeast is needed to substantiate it. To decide whether or not β-d-fructo-pyranose is fermented by yeast cells use was made of the fact that mutarotation of d-fructose proceeds at its minimum rate at 0° C. and at pH values between 3·2 and 5·1 (Nelson and Beegle, 1919). Even under these conditions mutarotation of β-d-fructo-pyranose is appreciable. Conditions were so arranged (sugar concentration not higher than 0·1 p.c. and pH = 4·3) that interconversion of fructo-pyranose to fructo-furanose was minute within the time of the experiment (40 minutes).

METHODS.

Baker's yeast (Effront), grown and matured during the preceding 16 hours and compressed 3 hours before the beginning of the experiment, was used; thus autofermentation during the experiments was reduced to an unmeasurable amount. Unless stated otherwise, 8·0 gm. yeast and 21·0 c.c. M/10 KH_2PO_4 solution were put into a centrifuge cup of 50 c.c. capacity and the stoppered cup with the well mixed yeast suspension balanced against a cup filled with water. The cup containing the suspension was then placed in a large thermos flask packed with snow and allowed to cool down to 0·1°–0·0° C. The thermos flask was kept in the cold room (the temperature of which varied between 0° C. and −1° C.), in which the experiments were performed. After equilibrating the yeast suspension and the gas-space above it with carbon dioxide (containing less than 0·1 p.c. oxygen) the sugar was added and dissolved and the gas-space once more equilibrated with CO_2. The pH of the suspension after equilibration with CO_2 was 4·3 at 18° C. After the addition of the sugar the cup was kept for 40 minutes in snow and the thermos flask containing it shaken every five minutes. At the end of the period it was centrifuged for 35 minutes

at 0° C. in a refrigerated centrifuge. The clear supernatant fluid was analysed for reducing substances and ethyl alcohol. In each experiment a control without sugar was treated in the same manner.

The α-d-glucose and β-d-fructose (pyranose) used in the investigations were recrystallized, anhydrous substances the specific rotations of which at time 0 and time ∞ were verified. The reducing power (R.P.) of fructose determined by the Hagedorn-Jensen micro-method was found to be 100 p.c. compared with glucose if the solution of fructose is submitted to the whole process of the method. R.P. is, however, 105·7 p.c. when the fructose solution is added to the filtrate of the zinc hydroxide solution after the latter had been heated for three minutes at 100° C. This agrees with the measurements of Sobotka and Reiner (1930).

The sugar left at the end of the experiment was determined in the clear supernatant fluid in triplicate (Hagedorn-Jensen) the results being calculated as glucose. The supernatant of the control did not contain appreciable amounts of reducing substances.

The determination of ethyl alcohol was performed as before (Gottschalk, 1941); the distillation of the alcohol, however, was preceded by deproteinization of the supernatant fluid with dialysed iron. In the controls and in the experiments with fructo-pyranose as substrate the bichromate solution used contained 0·050 gm. potassium bichromate in 100 c.c. conc. sulphuric acid and the final solution was titrated with N/200 sodium thiosulphate. The alcohol content of the control, on an average 1·5 mg. in the total volume, was subtracted from the value of the main experiment.

The dry weight of the yeast was determined as before (Gottschalk, 1942).

The calculation of the sugar consumption and alcohol production was made on the assumption that total water (external solution plus yeast water) is solvent water and that at the end of the experiment both the sugar added and the alcohol produced are uniformly distributed. This assumption is justified in view of the very low concentrations of sugar and alcohol involved and the experimental time.

EXPERIMENTAL RESULTS AND DISCUSSION.

TABLE 1.

Balance of the anaerobic fermentation of β-d-fructo-pyranose and α-d-gluco-pyranose at 0° C.

Dry weight of yeast added. gm.	Hexose added.	mg.	Hexose consumed. mg.	Alcohol produced. mg.	Glucose fermented. / Glucose consumed. p.c.
2·184	β-d-fructo-pyranose	20·3	1·8	0·5	
2·152	idem	20·0	1·8	0·6	
2·184	idem	20·2	1·7	0·5	
2·160	idem	20·0	1·8	0·7	
2·168	idem	20·2	2·0		
2·168	idem	20·2	2·1	0·7	
2·184	idem	20·8	2·0		
2·152	idem	19·8	1·8	0·4	
1·350*	idem	20·1	2·0		
1·365*	idem	20·0	1·8		
2·184	α-d-gluco-pyranose	20·6	15·0	3·9	50·8
2·152	idem	19·9	15·4	3·7	47·0
2·184	idem	20·2	14·7	3·6	47·9
2·168	idem	20·8	15·9	4·3	52·9
2·152	idem	20·0	15·5	4·0	50·5
1·350*	idem	20·8	10·2		

* 5·0 gm. fresh compressed yeast used instead of 8·0 gm.

Table 1 shows that at 0° C. and at pH = 4·3 of the suspension d-fructose, added to yeast in its β-pyranose form, is fermented only at a minute rate compared with α-d-glucose the fermentation of which proceeds at the expected rate taking into account the temperature coefficient of alcoholic fermentation. The minute rate of fructose consumption at 0° C. varying between 1·7 and 2·1 mg. is—in contrast to the rate of glucose consumption—independent of yeast concentration indicating the non-enzymatic nature of the rate-determining factor. This minute rate of fructose utilization can be accounted for if fructo-furanose is the fermentable fructose form and if the partial interconversion of d-fructo-pyranose to d-fructo-furanose is the controlling reaction. From the experimental data furnished by Nelson and Beegle (1919) and by Isbell and Pigman (1938) concerning the mutarotation of β-d-fructo-pyranose at 0° C. and at pH values between 3·2 to 5·1 it follows that after a period of 40 minutes about 56 p.c. of the total change in optical rotation of β-d-fructo-pyranose towards equilibrium has been accomplished. This change consists, as mentioned above, for the most part in a pyranose ⇌ furanose interconversion. Ex-

periments reported in the following paper (Gottschalk, 1943) prove that at 0° C. a d-fructose solution at equilibrium contains about 12 p.c. of the sugar in the furanose form. Therefore, the mutarotation of 20 mg. β-d-fructo-pyranose at 0° C. and pH = 4·3 will yield approximately 1·34 mg. fructo-furanose in 40 minutes. This calculation, disregarding the possibility of interconversion of the β- and α-fructo-furanoses and of interconversion of the β- and α-pyranoses, represents only a first approximation to the true figure. Its justification appears from the fact that with different initial concentrations of β-d-fructo-pyranose the rate constant $(k_1 + k_2)$ is invariable if calculated according to the equation $(k_1 + k_2)t = \ln \frac{a_e - a_o}{a_e - a}$, where a_o and a_e are the optical rotations at t = 0 and t = infinity (Hudson, 1909; Nelson and Beegle, 1919; Purves and Hudson, 1934; Isbell and Pigman, 1938).

Furthermore we must take into account the fact that the pH inside the yeast cell does not correspond to that of the surrounding medium determined as 4·3. Holden and I (cf. addendum) have shown that the actual pH of the yeast cell (Effront) is 5·9 and is strongly fixed by an efficient buffering system. This means that in experiments with 8 gm. fresh yeast 21·6 p.c. of the β-d-fructo-pyranose added mutarotates with a velocity constant $(k_1 + k_2) \times 10^2 = 1·04$ (pH = 6·0) and 78·4 p.c. with a velocity constant $(k_1 + k_2) \times 10^2 = 0·87$ (pH = 4·3). The application of this correction increases the calculated figure only from 1·34 to 1·40 mg. fructose.

The calculated figure of 1·4 mg. fructo-furanose holds only if the reversible reaction fructo-pyranose $\rightleftharpoons$ fructo-furanose is undisturbed by fermentation which interferes with the establishment of the equilibrium. Yeast, by fermenting the fructo-furanose produced in the course of mutarotation, cuts down the back reaction with the result that more fructo-furanose is available to the fermenting yeast. This explains why under the conditions of the experiment on an average 1·9 mg. fructose is fermented instead of 1·4 mg.

TABLE 2.

5·0 gm. fresh compressed yeast suspended in 23·0 c.c. M/10 KH_2PO_4 solution; saturated with CO_2 at 0° C. Temperature: 0° C. Gas-space: CO_2.

β-d-Fructo-pyranose added (mg.).	Fructose concentration in suspension fluid (mg. p.c.) after 0	10	15	40 minutes.
19·9	86·5	75		
20·0	87·0		73	
20·0	87·0			68

The results in Table 2 illustrate the decrease with time in the fructose concentration of the suspension fluid. During the experiments there was no volume change of the yeast cells. This was determined by haemotocrit (tube-size: internal diameter 7·8 mm.; length 9·88 cm.).

CONCLUSION.

d-Fructose, added in the crystalline state (β-pyranose) to a suspension of baker's yeast in M/10 KH_2PO_4 solution, is fermented at 0° C. only at a minute rate which is not controlled by an enzyme reaction. The rate of fermentation under the conditions described is almost identical with the rate of mutarotation by which part of the d-fructo-pyranose is converted into d-fructo-furanose. From these results the conclusion is drawn that d-fructo-pyranose is unfermentable by yeast. Fructo-furanose represents the fermentable fructose modification. This deduction is confirmed by a comparison of the rates of alcoholic fermentation by yeast at 0° C. with β-d-fructo-pyranose, with equilibrium d-fructose and with d-fructo-furanose (set free from sucrose) as substrates. The results of these experiments are reported in the following papers.

The obvious interpretation of the nonfermentability of β-d-fructo-pyranose is the lack of an OH-group attached to carbon atom 6. The presence of a primary alcohol group at carbon atom 6, as in fructo-furanose, is a prerequisite for alcoholic fermentation, the first step of which is the reaction between this OH-group and the mobile phosphate group of adenosinetriphosphate catalysed by hexokinase and resulting in the formation of the individual hexose-6-phosphate.

Mutarotation of α-d-glucose proceeds at 0° C. extremely slowly $(k_1 + k_2) \times 10^2 = 0·077$ at pH = 3·05 — 5·35, i.e. at less than one-tenth of the rate of mutarotation of β-d-fructo-pyranose. This fact in view of the observed rate of

glucose fermentation affords definite evidence that α-d-glucose is directly fermentable, i.e. without any preliminary change of its molecular structure. In this respect the suggestion of Hopkins and Roberts (1936) has to be revised.

In the experiments of Table 1 the alcohol production from α-d-glucose is rather small, the ratio $\frac{\text{glucose fermented} \times 100}{\text{total glucose consumed}}$ being about 50. In experiments at 25° C. with 1 p.c. initial d-glucose concentration and 3 p.c. fresh yeast this ratio was found to be 77 (Gottschalk, 1941). The lower value under the experimental conditions described here suggests that with the distribution of a very small amount of sugar among a large quantity of yeast the synthesis of polysaccharides—the main path of utilization of the glucose consumed but not fermented—increases at the cost of carbohydrate breakdown to carbon dioxide and alcohol. Dumazert and Penet (1938) have observed that with d-glucose concentrations as low as 0·09 p.c. the alcohol yield in the process of fermentation amounts only to about 45 p.c.

NOTE ON THE pH OF THE YEAST CELL.

The hydrogen ion concentration of fresh baker's yeast (Effront) was determined by three independent methods: (1) freezing in solid carbon dioxide and alcohol, (2) freezing in liquid air and (3) heating to the boiling point with an equal volume of distilled water. The results were (1): 5·92; (2): 5·94; (3): 5·90. They agree with those of the colorimetric determination of pH = 5·9 for top yeast XII by Mahdihassan (1930). In addition the buffering power of the yeast cell was measured and found to be high compared with that of serum (cf. Table 3). From measurements made on liquid centrifuged from the thawed yeast (which solution contained less than 1 p.c. protein), it was seen that the buffering power resides in the salts, the proteins playing little part.

TABLE 3.

50 gm. fresh baker's yeast frozen three times in liquid air; diluted with 50 c.c. distilled water.

Volume of N/22 H_2SO_4 added to 25·0 c.c. of the yeast suspension. c.c.	pH observed (by glass electrode).
0	5·94
1·0	5·85
5·0	5·59
10·0	5·27

The pH determinations were performed by Mr. H. F. Holden.

SUMMARY.

At 0° C. d-fructose, added in its β-pyranose form to a suspension of baker's yeast in M/10 KH_2PO_4 solution, is only fermented at a minute rate compared with α-d-glucose, the fermentation of which takes place at the expected rate. The minute rate of fructose consumption is independent of yeast concentration, i.e. not controlled by an enzyme reaction. The rate-determining reaction is rather the partial interconversion of d-fructose-pyranose to d-fructo-furanose in the process of mutarotation. The conclusion must be drawn that d-fructo-pyranose is unfermentable by yeast and that it is the furanose modification of fructose which undergoes alcoholic fermentation.

α-d-glucose is fermented without any preliminary change of its molecular structure.

The pH of the yeast cell was found to be 5·9.

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REFERENCES.

Dumazert, C. et Penet, G. (1938): Compt. rend. soc. biol., 127, p. 75.
Gottschalk, A. (1941): Austral. J. exp. Biol., 19, p. 211.
Gottschalk, A. (1942): *Ibid.*, 20, p. 173.
Gottschalk, A. (1943): *Ibid.*, 21, p. 139.
Hopkins, R. H. (1931): Biochem. J., 25, p. 245.
Hopkins, R. H. and Roberts, R. H. (1935a): *Ibid.*, 29, p. 931.
Hopkins, R. H. and Roberts, R. H. (1935b): *Ibid.*, 29, p. 2486.
Hudson, C. S. (1909): J. Amer. chem. Soc., 31, p. 655.
Isbell, H. S. and Pigman, W. W. (1938): J. Research Natl. Bur. Standards, 20, p. 773.
Mahdihassan, S. (1930): Biochem. Z., 226, p. 203.
Nelson, J. M. and Beegle, F. M. (1919): J. Amer. chem. Soc., 41, p. 559.
Purves, C. B. and Hudson, C. S. (1934): *Ibid.*, 56, p. 706.
Slator, A. (1908): J. chem. Soc., 93, p. 238.
Sobotka, H. and Reiner, M. (1930): Biochem. J., 24, p. 394.

THE PROPORTION OF FRUCTO-FURANOSE PRESENT IN d-FRUCTOSE SOLUTION AT EQUILIBRIUM (0° C.)

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From the biochemical point of view the determination of the composition of a sugar solution at equilibrium is of considerable interest if the components are of unequal value for the organism. This holds particularly for d-fructose. A solution of d-fructose at equilibrium contains both the pyranose and furanose modifications of the sugar besides open chain forms. In any quantitative consideration of the composition of d-fructose at equilibrium in acid solution the open chain forms can be neglected: the absorption coefficient obtained by the use of very concentrated d-fructose solutions (Bednarczyk and Marchlewski, 1938) is clear evidence for the absence of any measurable amount of the keto-form; the enol-form can be excluded because enolization does not take place appreciably in acid medium. In a previous paper (Gottschalk, 1943) it has been shown that β-d-fructo-pyranose, the main component of d-fructose at equilibrium, is unfermentable. The same holds for the small fraction of α-d-fructose-pyranose which may be present in a d-fructose solution at equilibrium; for it is evident that it is the (2, 6) position of the oxygen bridge with its effect on carbon atom six and not the β-isomerism at C_2 which renders the β-pyranose form unfermentable. Thus fructo-furanose remains as the only fermentable component of a d-fructose solution at equilibrium and its quantitative determination by the application of yeast has become possible.

EXPERIMENTAL.

To reduce to a minimum the partial interconversion of d-fructo-pyranose to d-fructo-furanose which takes place immediately after the removal by fermentation of the furanose form preformed in the equilibrium mixture, the experiments were performed at 0° C. and pH = 4·3.

The technique and the methods used were the same as in the previous paper (Gottschalk, 1943). To produce a d-fructose solution at equilibrium at 0° C. 20 mg. β-d-fructose (pyranose) were dissolved in 1·0 c.c. M/10 KH_2PO_4 solution and allowed to mutarotate for 22 hours at 0° C. This fructose solution was added to the yeast suspension cooled to 0° C. and equilibrated with carbon dioxide. The small vessel containing the sugar solution was washed out twice with 0·5 c.c. M/10 KH_2PO_4 solution at 0° C. and the washings added to the suspension. The sugar added to the control was β-d-fructose (pyranose) in the crystalline state.

Experiments 1–2 were carried out to obtain a rough estimate of the proportion of fructo-furanose present in a d-fructose solution at equilibrium at 0° C. Subsequently the amounts of β-d-fructose (pyranose) and d-fructose at equilibrium (0° C.) added to the yeast suspensions were so adjusted that in both cases the fructose concentrations at the end of the experiment were the same. By this procedure the quantity of β-d-fructo-pyranose added to the control experiment corresponded exactly to the amount of fructo-pyranose left in the main experiment after removal of the preformed fructo-furanose by fermentation. Since this removal takes from 3 to 4 minutes, as is concluded from experiments with small amounts of d-glucose as substrate, the β-d-fructose (pyranose) was added to the control 4 minutes after the beginning of the main experiment. In some controls to imitate the conditions of the main experiment as closely as possible 2·5 mg. α-d-glucose were added as well as β-d-fructo-pyranose. This addition was without any effect on the rate of fructose consumption in the control experiment.

RESULTS AND DISCUSSION.

TABLE 1.

5·0 gm. fresh compressed baker's yeast (Effront) suspended in 23·0 c.c. M/10 KH_2PO_4 solution; saturated with CO_2 at 0° C. (initial pH = 4·3 at 18° C.). Temperature: 0° C. Gas-space: CO_2.

No.	Time. min.	d-Fructose.	Added. mg.	Fructose consumed. mg.	Fructo-furanose preformed. Equilibrium fructose added. p.c.
1	40	β-pyranose	20·3	1·8	
	40	equilibrium	20·2	3·9	
2	40	β-pyranose	20·2	2·0	
	40	equilibrium	20·1	4·0	
3	36	β-pyranose	17·6	1·8	
	40	equilibrium	20·0	4·2	12·0
4	36	β-pyranose	18·0	1·4	
	40	equilibrium	20·6	4·0	12·6
5	36	β-pyranose	17·9	1·7	
	40	equilibrium	20·5	4·3	12·7
6	36	β-pyranose	17·6	1·6	
	40	equilibrium	19·9	3·9	11·6

The experimental results (Table 1) show that a solution of d-fructose at equilibrium at 0° C. (pH = 4·3) contains about 12 p.c. of the sugar in the furanose form. According to Ohle (1927) the furanose form present in d-fructose solution after mutarotation is almost exclusively β-d-fructo-furanose.

The proportion of fructo-furanose present in an equilibrium mixture changes with temperature. For a long time it has been known that the specific rotation of d-fructose at equilibrium varies with temperature due to a change in the relative proportions of the individual components of the solution. Increase in temperature shifts the equilibrium towards the less laevorotatory forms as expressed by the formula of Vosburgh (1920): $[\alpha]_D^t = [\alpha]_D^{25} + (0{\cdot}566 + 0{\cdot}0028c)\ (t - 25)$ (valid for temperatures between 15° and 37° C.).

An estimate based on the specific rotations of d-fructose at equilibrium at 0° C. and at 20° C. and on the data of this paper suggests that a d-fructose solution at equilibrium at 20° C. contains approximately 20 p.c. of the hexose in the furanose modification. This rather large proportion of fructo-furanose present in an equilibrium mixture of d-fructose at 20° C. taken together with the high mutarotation constant of β-d-fructo-pyranose at 20° C.—$(k_1 + k_2) \times 10^2 = 14{\cdot}5$ at pH = 6·92—explains why the velocity of the biological utilization of a fructose solution is seldom limited by the fact that only part of the sugar is able to combine directly with the cell enzymes.

SUMMARY.

A solution of d-fructose at equilibrium at 0° C. (pH = 4·3) contains about 12 p.c. of the sugar in the furanose form; at 20° C. this proportion amounts probably to approximately 20 p.c.

REFERENCES.

Bednarczyk, Wl. and Marchlewski, L. (1938): Biochem. Z., 300, p. 42.
Gottschalk, A. (1943): Austral. J. exp. Biol., 21, p. 133.
Ohle, H. (1927): Ber., 60, p. 1168.
Vosburgh, W. C. (1920): J. Amer. chem. Soc., 42, p. 1696.

CAPILLARY FAT-EMBOLISM IN THE BRAINS OF SHEEP, PIGS AND MONKEYS, WITH ESPECIAL REFERENCE TO DEMYELINATION AND OTHER LESIONS IN THE WHITE MATTER[1]

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A simple domestic "mayonnaise" injected into the common carotid artery of rabbits, after ligation of the external carotid, penetrates readily to the cerebral capillaries. The globules excite surprisingly little reaction on the part of nervous or vascular tissues (Hurst and Cooke, 1942). Cooke, Hurst and Swan (1942) used the obstructing globules to deposit a virus in a selected part of the nervous system. In the present work we sought to determine the action of haemolysins, precipitins and neurotoxic antibodies similarly carried to the brain. Perhaps because of insufficient adsorption and concentration on the surface of the globules of the antibodies thus introduced, our experiments did not demonstrate a specific effect. They appear, however, to throw light on the manner in which the white matter is involved under experimental conditions created by ourselves and others, and in particular on the susceptibility of the myelin in certain pathological processes.

EXPERIMENTAL WORK.

Most of the experiments were performed on sheep, a limited number on pigs and monkeys.

Sera. We used:

(i) normal rabbit serum (sheep) or normal sheep serum (pigs).

(ii) rabbit serum haemolytic for sheep red-blood-corpuscles to a titre of 1: 12,800.

(iii) serum of rabbits injected repeatedly with sheep serum. After absorption of haemolysins, a 1: 50 dilution of antiserum tested against falling dilutions of antigen gave an end-point of 1: 8,000 in the precipitin test.

(iv) "neurotoxic" serum from rabbits injected intramuscularly with 6·7 gm. sheep brain in the course of 55 days and allowed to rest for a further 14 days. The serum was absorbed repeatedly with red blood corpuscles, or with red cells and splenic suspensions, until the haemolytic titre was less than 1: 10. Injected intrathecally in doses of 5 c.c. into sheep it evoked symptoms of meningeal irritation. Histologically, meningeal infiltration with polymorphs and mononuclears (1 day) or with mononuclears (3 and 6 days) resulted from its use.

Preparation of "mayonnaise". We have already described the preparation of this emulsion (Hurst and Cooke, 1942). Greater experience at different times of the year has shown that in warm weather it is necessary thoroughly to cool the ingredients and to grind them in a chilled mortar in order to obtain small globules of relatively uniform size. Even in cool weather the best results follow the use of materials direct from the cold room. In the experiments on sheep we used one of three preparations. For mayonnaise A we ground 5 c.c. egg-yolk with 2·5 c.c. olive oil until globules of the requisite size were obtained; we then added 2·5 c.c. egg-albumen with further short grinding, and finally 5 c.c. of the serum to be tested. For mayonnaise B (haemolytic serum) we added 0·25–1·0 c.c. of the serum immediately after grinding the yolk and olive oil, then the egg-albumen, and finally 4–4·75 c.c. of saline; with the larger doses of serum, blood-pigment often appeared in the faeces within the 24-hours following administration. Mayon-

[1] This work was performed with a grant from the National Health and Medical Research Council.

naise C was prepared like mayonnaise B except that serum was used for the final dilution. In all cases we controlled the size of the globules by micrometry.

Technique of injection. In sheep the equivalent of the internal carotid artery originates from a branch of the external carotid in a surgically inaccessible region near the base of the skull. The common carotid gives off a number of branches to various structures in the neck. We injected the main artery at a point deep to and slightly above the level where the submaxillary gland approaches nearest to the thyroid. Here the artery may be reached with a minimum of dissection, and most of the inconvenient branch vessels be avoided, through an antero-posterior incision bisecting a line joining the angle of the jaw and the lower margin of the larynx. We injected 5 c.c. of the emulsion into the right carotid artery while the blood was flowing through; we did not find it necessary to use clamps or ligatures. The needle was of gauge 23 and the injection complete in about 20 seconds.

The pig possesses an internal carotid artery. As in the sheep, however, we found it convenient to inject a large amount of mayonnaise into the common carotid without clamping the external carotid, rather than to give a small amount all of which would traverse the internal carotid.

The monkeys received 0·5 c.c. mayonnaise (without serum) into the common carotid after ligation of the external carotid.

We killed the sheep from 1 to 130 days after inoculation, the pigs after 3 or 12 days and the monkeys within 3 to 10 days.

RESULTS.

Effects in Sheep of Serum-mayonnaise.

The brains of all injected animals contained fatty globules lying almost exclusively in the capillaries, and very largely on the side of the inoculated carotid artery. The globules had adapted themselves to their surroundings, and often formed elongated plugs extending for some little distance along a vessel. They were far more abundant in the grey than in the white matter. In general, they were numerous in animals killed within a day or two of injection; thereafter they became less plentiful, and after from one to several weeks, the few remaining stained feebly with scharlach R or showed vacuolation or incomplete contours. Only seldom did slight swelling and multiplication denote a reaction to their presence on the part of the endothelium (cf. Hurst and Cooke, 1942). Even where closely adjacent capillaries were occluded, or where occasional globules occupied the lumina of pre-capillaries, large areas of the parenchyma in all animals permanently escaped damage.

In addition to fatty globules, nine brains contained much more voluminous, spherical or elongated plugs distending the capillaries and sometimes spreading from these into the arterioles and venules (Figs. 5 and 6). Usually they were not numerous. In the larger vessels they frequently lay to one side of the lumen, only partly obstructing it. These plugs stained deeply with haematoxylin or showed deeply staining spherules embedded in a lighter matrix; in haematoxylin and scharlach preparations some coloured also with the fat stain, appearing then reddish-purple in tint. They were hyaline without internal structure, and never possessed the staining reactions of fibrin or the appearance of an ordinary thrombus. In some capillaries they could be seen flanking on each side a fatty globule; their free ends were covered with sparse polymorphonuclears or mononuclears. Others occurred independently of fatty globules. In the vicinity of most the nervous tissues showed no abnormality, in spite of the obvious distension and obliteration of the small vessels. It will be noted (Table 1) that these plugs were detected only in animals killed shortly after injection; the inference is that like the fatty globules they disappeared as time elapsed. We obtained no evidence as to the precise mechanism of their disappearance; in particular we saw no signs of organization or of their removal by phagocytes.

In the brains of some animals no parenchymatous lesions were detected; at most slight perivascular cuffing was fairly widespread over the hemisphere of the inoculated side. Even in some brains containing the more voluminous plugs no lesions were present. On the other hand, if examined at a sufficiently early stage, all brains exhibiting definite changes showed, in relation to the individual lesions, either plugs, or exceptionally large fatty globules, or a concentration of capillaries blocked by smaller globules. In view of the observations mentioned above, the absence of globules or plugs from some lesions observed at a later stage is of course not significant. The lesions were often extremely small and almost impossible to detect with the naked eye on the cut surface of the brain. When, however, this was frozen on the head of the microtome, they became very prominent as tiny dark specks on the cream-coloured background.

The ensuing description summarizes very briefly the state of affairs in animals killed after the first few days. In spite of far more numerous capillary emboli, the grey matter rarely showed areas of complete necrosis; "partial damage", with disappearance of nerve cells, proliferation of microglia, and reactive changes in the neuroglia and vessel walls, occurred much more frequently. In the white matter every degree of change was seen, from areas of "overcellularity" not unlike those seen in early poisoning with KCN (Hurst, 1940, 1942) and accompanied by ballooning and fragmentation of individual myelin sheaths, through areas from which myelin was almost or wholly absent but in which the nuclei of interstitial cells stained normally, to areas of complete necrosis. The last were rare and were surrounded usually by a wider zone of less

complete destruction. Serial sections showed that areas of "partial destruction" did not represent necessarily the edges of foci of more severe change, nor were they collections of fibres interrupted elsewhere by a necrotic focus and undergoing secondary degeneration. The myelin sheaths suffered one of two changes. In the smaller (and possibly more slowly developing) areas of severe change they were distorted considerably in outline, showing much ballooning and fragmentation but staining deeply with haematoxylin. In the larger areas of complete necrosis, those in the centre retained their normal form, though not their tinctorial properties, for a much longer time (cf. Hurst, 1940). In all cases the axis-cylinders suffered equally with the myelin sheaths. In the later stages necrotic areas were replaced by massed granular corpuscles seldom intermingled with collagen fibrils, while in less affected areas the microglia was largely polymorphic and glial sclerosis marked. In both cases the vessels showed the usual reactive changes. Few of the areas possessed very sharp or definite margins; in serial sections they could be followed for considerable distances (up to 1·5 mm.), altering their outlines and giving off ramifications at different levels. Demyelination was never strictly perivascular. In exceptional animals a very few small ring haemorrhages were present.

Eight of 13 sheep receiving normal serum—mayonnaise, 12 of 15 haemolytic serum—mayonnaise, 6 of 6 precipitating serum—mayonnaise, and 9 of 19 neurotoxic serum—mayonnaise exhibited such lesions (Table 1) (Fig. 1). No qualitative differences in the lesions could be attributed to the use of a particular serum. Within the group of sheep receiving neurotoxic serum, it seemed that the development of especially severe lesions (accompanied by symptoms such as grinding of the teeth, circling to the left, deep breathing, apathy, spasms, rigidity, etc., and lasting for 24–72 hours after inoculation) was associated with a particular method of preparing the emulsion when using a serum absorbed with splenic substance. In none of these cases were ordinary thrombi present. In the various experiments the animals were inoculated in batches of three or four; to ensure even spread of variations due to differences in successive preparations of mayonnaise, we chose from each batch sheep for short- and long-term observation.

TABLE 1.

Lesions in sheep injected with serum—mayonnaise.

Sheep.	Days after injection.	Fat in cerebral capillaries.	Plugs in cerebral capillaries.	Lesions in grey matter.	Lesions in white matter.
		Normal rabbit serum—mayonnaise A.			
556	1	++	0	0	0
435	2	++	±	0	0
440	4	+	0	±	++
437	8	+	0	±	+
444	10	+	0	+	+
442	15	+	0	+	++
443	22	+	0	+	+
445	30	±	0	+	+
439	60	±	0	0	0
438	87	±	0	0	+
441	130	±	0	0	0
		Normal rabbit serum—Mayonnaise C.			
273	9	±	0	0	0
274	9	±	0	0	+
		Neurotoxic rabbit serum absorbed with red blood corpuscles—Mayonnaise A.			
446	1	++	0	0	0
447	4	+	0	0	0
451	7	±	0	0	0
448	15	±	0	0	0
452	22	±	0	0	0
449	60	±	0	0	0
		Neurotoxic rabbit serum absorbed with red blood corpuscles and spleen—Mayonnaise A.			
453	2	++	±	0	0
457	4	+	±	0	0
455	7	+	0	0	0
458	10	±	0	0	±
459	22	±	0	0	±
454	62	±	0	0	0
456	90	±	0	0	±

TABLE 1 (continued)

Sheep.	Days after injection.	Fat in cerebral capillaries.	Plugs in cerebral capillaries.	Lesions in grey matter.	Lesions in white matter.
Neurotoxic rabbit serum absorbed with red blood corpuscles and spleen—Mayonnaise C.					
467	1	++	++	+	+
468	4	±	±	+	++
470	7	±	0	+	+
471	15	±	0	+	+
472	22	±	0	+	++
469	30	±	0	+	+
Haemolytic rabbit serum—Mayonnaise B.					
477	1	+	±	±	±
484	1	++	+	0	0
488	3	+	±	+	++
489	7	+	0	++	+
491	10	+	0	±	+
481	10	+	0	±	0
474	10	+	0	+	+
480	13	±	0	0	±
492	13	±	0	0	±
487	15	±	0	0	0
490	22	±	0	±	++
482	30	±	0	±	+
493	57	±	0	0	±
483	60	±	0	0	±
486	90	±	0	0	0
Precipitating rabbit serum absorbed with red blood corpuscles—Mayonnaise C.					
495	2	+	±	+	++
496	10	±	0	+	++
497	10	±	0	±	0
499	14	±	0	±	±
494	22	±	0	+	+
498	30	±	0	0	+

In the white matter the largest lesions covered areas up to 1·5 mm. in longest diameter, even though serial sections demonstrated that only seldom were vessels other than capillaries blocked. Although they were not very much more numerous in the white than in the grey matter, on an average lesions were far larger and more severe in the former. In determining the number of lesions in different parts, we examined sections from three levels in each of 34 brains taken at random. One section passed vertically through the anterior frontal region, one through the anterior part of the corpus striatum (missing the globus pallidus), and one through the optic thalamus and region of entry of the optic tracts. We classed the lesions arbitrarily as large, small and tiny. In the white matter we counted 66 large, 54 small and 1 tiny, in the grey matter 43 large, 43 small and 18 tiny. Of the lesions in the grey matter, 69 lay in the cerebral cortex, especially in parts near the vertex, 25 in the corpus striatum, and 10 in the optic thalamus. Those in the white matter occupied situations similar to those affected in poisoning with KCN. They lay centrally in the hemisphere, in the corpus callosum, or in the tongues of white matter projecting into a gyrus (Fig. 1). In the last position they were often elongated in the long axis of the gyrus, and nearly always a band of intact white matter separated the damaged area from the overlying cortex. Very rarely an elongated focus lay with one extremity in the cortex and one in the underlying white matter, but ordinarily the lesions occurred either in the cortex or in the white matter without transgressing the boundary between the two. A few areas lay in the contralateral hemisphere a short distance from the middle line. No lesions were seen in the optic tracts, structures commonly affected in poisoning with NaN_3 and KCN (Hurst, 1940, 1942).

Effects in Sheep of Mayonnaise without Serum.

These were essentially the same as those described above. As the volume of mayonnaise injected was increased (up to 30 c.c.), the animals were left with permanent nervous symptoms among which marked apathy was the rule. Lesions in the grey matter, and particularly in the cerebral cortex, were more numerous absolutely and relatively; the great majority still showed only partial destruction of the tissues. Those in the white matter were often much larger and included full necroses of large tracts of tissue (Fig. 2). The density of fatty globules caught in the capillaries was of course much greater than in the experiments with serum—mayonnaise. No large vessels were obstructed. Capillary plugs were seen in one animal killed at an early stage.

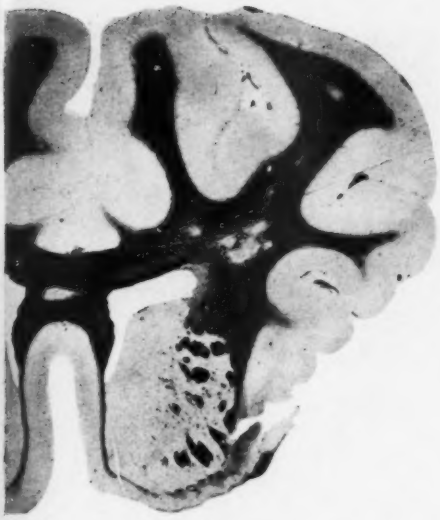


Fig. 1.

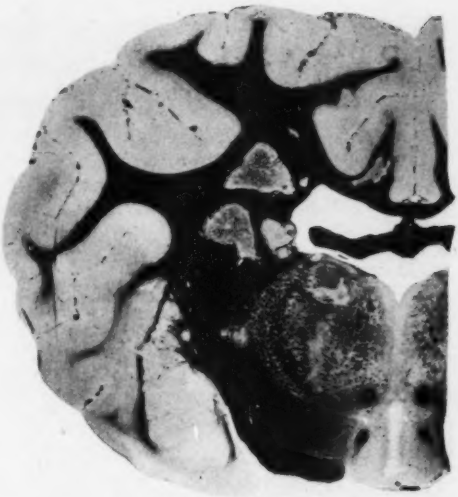


Fig. 2.

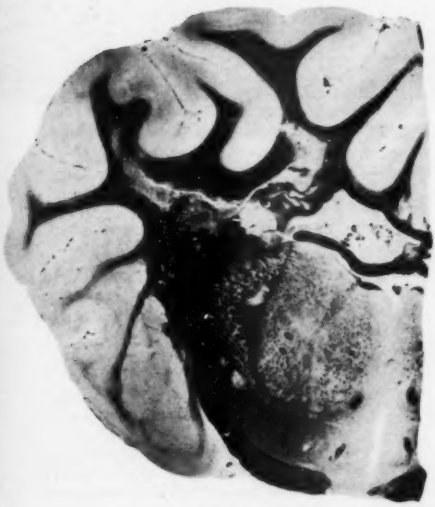


Fig. 3.

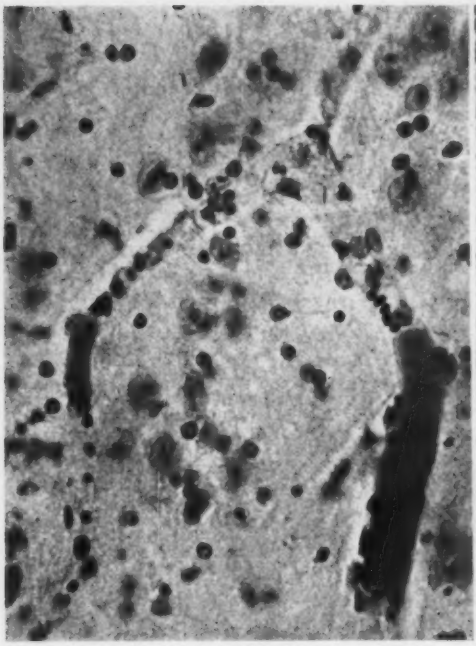
Fig. 4.

Fig. 1. Areas of demyelination in the white matter of a sheep receiving serum—mayonnaise. Weil's stain.

Fig. 2. Massive areas of necrosis in the white matter of a sheep receiving a large amount (15 c.c.) of mayonnaise without serum. One small area (above) extends into the cortex. There is a large area of necrosis in the optic thalamus, but for the most part areas of damage to the grey matter though numerous do not amount to full necrosis of tissue. Weil's stain.

Fig. 3. Massive softening of much of the white matter of the hemisphere in a sheep receiving a suspension of kieselguhr. There existed many areas of necrosis and of "partial damage" in the grey matter of the cortex and elsewhere. Weil's stain.

Fig. 4. Areas of necrosis and of demyelination in the white matter of a pig receiving serum—mayonnaise. Several extend from the white into the grey matter. Areas of damage to the grey matter are more numerous than in sheep similarly treated and some can be seen faintly in the cortex at the vertex. Weil's stain.



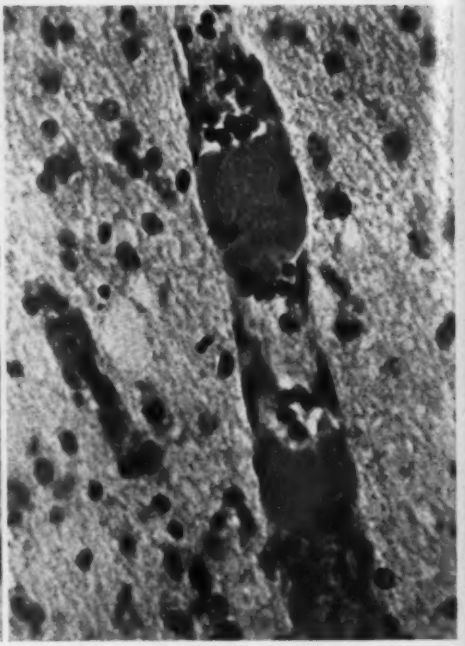


Fig. 5. **Fig. 6.**

Fig. 5. Basophilic plugs (not thrombi) distending a capillary (left) and a slightly larger vessel (but one without a definite wall other than endothelium) in a sheep receiving serum—mayonnaise 1 day previously. Note the absence of appreciable changes in the surroundings. Iron alum-haematoxylin and van Gieson.

Fig. 6. Plugs greatly distending a vessel slightly larger than the average capillary (but having no wall other than endothelium) in a pig receiving serum—mayonnaise 3 days previously. In the original preparation it is clear that these plugs are not ordinary thrombi. Note the absence of appreciable changes in the surroundings and the presence of a few leucocytes applied to the ends of the plugs. Iron alum-haematoxylin and van Gieson.

Effects of Kieselguhr in Sheep.

The mechanism by which particles of washed kieselguhr block vessels of comparatively large size has been mentioned elsewhere (Hurst and Cooke, 1942). The injection into one carotid artery of from 3 to 5 c.c. of a 1: 60 suspension produced in three sheep massive areas of complete necrosis in the grey or white matter, or involving both (Fig. 3).

Lesions in Pigs.

Of 8 pigs receiving normal serum—mayonnaise, only one showed a density of capillary emboli comparable with that commonly found in sheep. Five of the brains were normal. The remainder exhibited lesions comparable with those in sheep. These were, however, relatively more numerous in the grey matter, though as in sheep the lesions in the white matter were the larger and more severe. They showed a rather greater tendency to transgress the boundary between white and grey matter (Fig. 4). Capillary plugs were present in two animals killed on the third day; they were less basophilic than those in sheep. In the pig most severely affected ring haemorrhages occurred fairly frequently.

Lesions in Monkeys.

Three monkeys receiving mayonnaise without serum presented changes essentially similar to those in pigs. Basophilic capillary plugs were present in the brain of an animal killed on the third day.

DISCUSSION.

Russell (1941) and Greenfield (1941) have described the nervous lesions following fat-embolism in man. The former mentioned the frequent absence of parenchymatous change in the vicinity of blocked capillaries; she found more numerous lesions in the grey than in the white matter. Vascular thromboses and ring haemorrhages contributed to the nervous damage. Greenfield observed also perivascular demyelination around vessels plugged with fat or thrombus. "The tissue in most of these zones could not be called necrotic, since there was little alteration of nuclei; the myelin however failed to stain. . . . The axis cylinders . . . . in the demyelinated area . . . . showed evidences of degeneration." In a few areas there was central necrosis. The macroscopic appearances gave no hint of the existence of the areas, but as we have recounted above they may be seen readily when the tissue is frozen. Some of the areas extended from the white matter into the cortex. The areas of destruction of neurons and myelin were "quite unlike those seen in any other condition". In Greenfield's case as in our sheep haemorrhages were insignificant.

Our own series of observations seem to show that lesions follow relatively minor disturbances of the cerebral circulation in areas (1) where many closely adjacent capillaries are blocked by small globules of fat, (2) in the vicinity of some exceptionally large globules, and (3) where the capillaries and rather larger vessels become obstructed by the peculiar hyaline plugs we have described. It should be emphasized that these plugs are not ordinary thrombi, which were absent from all our cases save one pig. Whether they are formed solely in response to the introduction of a foreign protein, and hence are likely to be wanting in human cases, we cannot say. An important point, however, is that both these and many of the fatty globules apparently disappear from the brains during the first few days after injection of mayonnaise, so that examination of a case of fat-embolism of some duration does not suggest the full magnitude of the vascular obstruction initially sustained.

In most of our experiments in sheep lesions in the white matter were more numerous than those in the grey. With the largest doses of mayonnaise the reverse seemed to be true, as also in pigs and monkeys. Whatever the number of lesions, those in the white matter were on an average nearly always larger and more severe, as might perhaps be expected when the relatively sparse blood-supply of the white matter is considered. Full necrosis of tissue was relatively uncommon; more often demyelination with proportionate destruction of axis-cylinders obtained. The distribution of lesions in the sheep, and often in the pig and the monkey, closely resembled that of the changes resulting from repeated poisoning with KCN (Hurst, 1940, 1942); these also ranged from slight demyelination to full necrosis according to the severity of the repeated bouts of anoxia. The present observations support the view already expressed that demyelination of the type under discussion is the response of the white matter to insults short of those causing full necrosis.

We made the foregoing experiments more with the object of conveying to the brain possibly myelolytic substances than from interest in the effects of fat-embolism *per se*. As stated, this object was not realized. The results may, however, be considered in relation to the belief of a number of writers that vascular blockage is the basis of various demyelinating diseases in man. Putnam, McKenna and Morrison (1931), Putnam (1935) and Hoefer, Putnam and Gray (1938) found that demyelination, with or without destruction of the axons, follows experimental obstruction of the small vessels, and particularly the venules, with fat or thrombus. On the

whole it appears unlikely that the lesions they produced really tallied with those in naturally occurring human diseases. In disseminated sclerosis and disseminated encephalomyelitis, Putnam (1936, 1937), Putnam and Alexander (1939) and Scheinker (1943) emphasized the occurrence of thrombi in the smaller veins and saw in the phenomenon the basis for the demyelination. Simon and Solomon (1935) and Simon (1942) found in disseminated sclerosis an increased coagulability of the blood when the patients received injections of adrenalin or typhoid vaccine. Marburg (1942), however, regarded phlebothrombosis as only one of the aetiological factors of the demyelination in disseminated sclerosis, while Dow and Berglund (1942) considered that the thrombi might result from local absorption of thromboplastic substances released by the breakdown of myelin. Many authors, of course, have failed to detect thrombi in cases of disseminated sclerosis. This failure Putnam (1937) believed to be due to difficulty in demonstrating certain thrombi even when present, and also to their rapid disappearance from the lesions. Dow and Berglund questioned the facile disappearance, without trace, of thrombi which had existed long enough to cause demyelination. The present observations suggest that certain types of vascular plug (not thrombi) may disappear within a few days from lesions to the formation of which they have obviously contributed. Whether this finding has any application in human pathology can be determined only by further study.

SUMMARY.

Introduced into the cerebral circulation of sheep, pigs and monkeys, globules of olive oil in the form of a mayonnaise may block many of the cerebral capillaries without causing damage to the parenchyma. In many animals, however, lesions develop in some areas where (1) many closely adjacent capillaries are blocked by small globules of fat, (2) an unusually large globule lodges, and (3) the capillaries and rather larger vessels become obstructed by peculiar hyaline plugs. These plugs are not ordinary thrombi; apparently they disappear within a very few days. The obstructing globules of fat also disappear progressively, so that examination of a brain in any but the earliest stages does not give a true idea of the number of vessels initially blocked. Lesions in the white matter of the brain are more numerous and/or more severe than those in the grey; the former range from areas in which there is damage to only a few myelin sheaths and axons to those in which full necrosis of tissue has occurred. Their distribution is similar to that of the areas of demyelination or necrosis in poisoning with KCN. These findings are discussed briefly in relation to recent observations on fat-embolism and on disseminated sclerosis and disseminated encephalomyelitis in man.

No evidence was obtained that haemolysins, neurotoxic antibodies or precipitins could be carried to the nervous system on the obstructing globules, at any rate in effective concentrations.

REFERENCES.

Cooke, B. T., Hurst, E. W. and Swan, C. (1942): Austral. J. exp. Biol., 20, p. 129.
Dow, R. S. and Berglund, G. (1942): Arch. Neurol. Psychiat., 47, p. 1.
Greenfield, J. G. (1941): Proc. Roy. Soc. Med. London, 34, p. 650.
Hoefer, P. F. A., Putnam, T. J. and Gray, M. G. (1938): Arch. Neurol. Psychiat., 39, p. 799.
Hurst, E. W. (1940): Austral. J. exp. Biol., 18, p. 201.
Hurst, E W. (1942): *Ibid.*, 20, p. 297.
Hurst, E. W. and Cooke, B. T. (1942): *Ibid.*, 20, p. 125.
Marburg, O. (1942): J. Neuropath. exp. Neurol., 1, p. 3.
Putnam, T. J. (1935): Arch. Neurol. Psychiat., 33, p. 929.
Putnam, T. J. (1936): Ann. int. Med., 9, p. 854.
Putnam, T. J. (1937): Arch. Neurol. Psychiat., 37, p. 1298.
Putnam, T. J. and Alexander, L. (1939): *Ibid.*, 41, p. 1087.
Putnam, T. J., McKenna, J. B. and Morrison, L. R. (1931): J. Amer. med. Assoc., 97, p. 1591.
Russell, D. S. (1941): Proc. Roy. Soc. Med., London, 34, p. 645.
Scheinker, I. M. (1943): Arch. Neurol. Psychiat., 49, p. 178.
Simon, B. (1942): *Ibid.*, 48, p. 509.
Simon, B. and Solomon, P. (1935): *Ibid.*, 34, p. 1286.

NUCLEAR INCLUSIONS DEVELOPING IN THE KIDNEYS OF AUSTRALIAN OPOSSUMS MAINTAINED UNDER LABORATORY CONDITIONS

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Intranuclear inclusions are so characteristic of many diseases caused by viruses that their presence in apparently healthy animals has led to presumptive diagnosis of a latent virus infection. In the case of the salivary glands of guinea-pigs the diagnosis is well-founded, but in most instances no supporting experimental evidence has been adduced. In the course of other work using Australian opossums (*Trichosurus vulpecula*) we observed peculiar intranuclear inclusions in the cells of some of the renal tubules. In the earlier stages of their development they resembled so closely the inclusions of certain virus diseases that our first thought was to transmit the condition by inoculation of "infected" kidneys. For the reasons stated in this paper, however, we did not succeed in determining their origin and nature.

SITUATION AND MORPHOLOGY OF THE INCLUSIONS.

The inclusions occurred chiefly in the convoluted tubules, but on occasion in the tubules of Henle especially in their ascending limbs. In the former as a whole, never more than about 10 p.c. of the cells were affected. At times, however, all those seen in the cross-section of a single tubule showed inclusions, particularly of tubules situated immediately beneath the capsule of the kidney. In the early stages of development, the inclusions were usually spherical and from 1–5 μ in diameter. Larger bodies attained a diameter of from 10–12 μ, or even (in a greatly hypertrophied nucleus) 20 μ; they tended to be less completely spherical in outline. The largest occurred in a kidney which showed much interstitial fibrosis, lymphocytic infiltration, and destruction and dilatation of tubules. In many animals, however, quite large inclusions were unassociated with other pathological changes, while occasionally fibrosis was found in the absence of inclusions.

The earlier forms, particularly when strongly acidophilic, were those most closely resembling the inclusions in virus diseases; on the whole they tended more consistently to be round and "disc-like" than those caused by viruses. Their smallest representatives often existed side by side with the nucleoli, and we obtained no evidence that they arose from degenerative changes in these structures. The nuclei containing them were of normal size or rather enlarged, with or without a concentration of the basichromatin on the nuclear membrane and a clear space around the inclusion. Inclusions of larger size were round, oval or irregular in shape. At times they were surrounded by an unstained space. Often, however, they acquired an "envelope" of basichromatic material connected by radiating strands with the general basichromatin of the nucleus. In such cases the last was

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[2] Working with grants from the Commissioners of Charitable Funds and the University of Adelaide.

large and hyperchromatic, and without knowledge of their earlier stages the inclusions might have been accepted as greatly hypertrophied and acidophilic nucleoli. Affected cells rarely suffered necrosis, but sometimes they desquamated into the lumen of the tubule. Some kidneys contained many large and hyperchromatic nuclei in which no inclusions were present. All kidneys harbouring advanced inclusions exhibited also numerous early forms.

Stained with iron-alum haematoxylin and eosin after fixation in Zenker-acetic acid, the bodies were usually definitely eosinophilic, but occasionally amphophilic. On the other hand with phloxine-methylene blue most, if not all, were often markedly basophilic; in one case only were they exclusively acidophilic. The largest often showed a ring of acidophilic material covering a basophilic core; the whole might be surrounded by the envelope of nuclear basichromatin mentioned above. With Mann's methyl blue-eosin after fixation in sublimate-formol, the inclusions stained pale red to deep crimson, depending on their size, and often possessed a dark red or deep blue rim at the periphery. Some of the largest appeared to consist of concentric layers differing in the intensity of their colouration with eosin. The Feulgen reaction was always negative.

OBSERVATIONS ON THE DEVELOPMENT OF THE INCLUSIONS.

I. The first group of 12 opossums came from varied sources. Some were captured at the local Zoological Gardens or elsewhere, and were examined within a few days or weeks of their arrival in the animal house. Professor Goldby of the Department of Anatomy kindly gave us others; these probably had been in captivity for relatively long periods. We kept no record of the origin of individual animals. Of the 12, 5 showed no inclusions, 4 many early inclusions, and 3 many advanced forms.

II. With the second group of 15 opossums, we kept exact records of the length of their sojourn in the animal house. Ten kept in captivity for from 2 to 31 days showed no inclusions. The same or other opossums after captivity for from 19 to 86 days showed fewer or more inclusions; in general the advanced forms appeared more frequently in those kept longest. Subsequently, not one of 52 animals recently arrived in the animal house exhibited inclusions (see below).

Two animals free from inclusions, as determined by preliminary unilateral nephrectomy, received by cardiac puncture 1 c.c. of a 10 p.c. suspension of affected kidneys. These were preserved at — 20° C. in 50 p.c. buffered glycerin in normal saline for a few days, while sections were made to determine whether or not they contained inclusions. Despite the fact that the suspensions were prepared with normal, unfiltered opossum serum (see Hurst, 1937), both animals died within a few minutes.

Three animals free from inclusions received intramuscularly, on two occasions, 1 c.c. of a 10 p.c. suspension of affected kidneys. When killed 7–38 days after the first injection, 2 of 3 showed many early inclusions. This apparently positive attempt at transmission was offset by the fact that 2 animals free from inclusions and kept for a further 55–64 days in the strictest isolation developed these bodies. Two others showing very early inclusions 23 and 37 days respectively after admission to the animal house had developed advanced inclusions after a further 22 and 49 days.

Attempts to grow a virus on the chorio-allantoic membrane of developing hens' eggs failed. Three experiments gave negative results after 5, 6 and 7 serial passages at 4–6 days' interval (usually 6 days). The three series were then pooled and 3 further passages made without positive result.

III. These observations suggested that the appearance of inclusions depended on the length of the animals' stay in captivity. We considered, therefore, whether the diet administered could be responsible. None of a number of colleagues consulted was able to state precisely what constitutes a "normal" diet for the opossum. We remembered that Smith (1930) had demonstrated the formation of intranuclear inclusions in the kidneys of calves fed from birth on cow-serum in place of the normal colostrum. Reference to the sections he had given us showed bodies resembling to some extent the earliest inclusions present in opossums. The resemblance was not exact, but it was more nearly so than Smith's description and illustrations suggest. Our animals had been fed on a diet consisting of rolled oats, pollard, crushed maize, meat meal, crude salt, apples and tap-water.

We used 24 opossums in an attempt to determine the effect of different diets. They were collected in 7 different parts of the city and environs, from near the centre to a point in the country about 40 miles distant. On their arrival at the laboratory we performed unilateral nephrectomy,

and found the kidneys of all to be free from inclusions. The animals received one of three diets. The first contained a proportion of animal protein, the second was a relatively protein-free diet, and the third a diet rich in vegetable proteins. The addition of honey was necessary to induce the animals to partake freely of the second and third.

(a) Eight opossums each received daily 1½ oz. of a mixture of equal parts of rolled oats, pollard and crushed maize moistened with milk, 1½ oz. minced meat, ½ apple, ½ orange, ½ oz. honey and abundant water. After 60–75 days on the diet, 4 showed many early inclusions and 3 none. After 150 days, 1 showed many advanced inclusions.

(b) Ten opossums received daily 2 apples, ½ carrot, 4 leaves of silver beet, ½ orange, ½ oz. honey and water. These animals lost weight considerably. Eight showed many early inclusions after 48–86 days, 1 advanced inclusions after 57 days, and 1 no inclusions after 50 days on the diet.

(c) Six opossums received unlimited crushed haricot beans and peanuts together with ½ apple, ½ orange, ½ oz. honey and water daily. Five showed many early inclusions after 46–66 days, 1 no inclusions after 50 days.

In these series of animals, in addition to the inclusions in the kidney, two showed a few very early forms in the hepatic cells and one in some of the glandular epithelial cells of the small intestine.

Six rabbits fed on diet (a) showed no inclusions, neither did our stock rabbits, guinea-pigs and mice maintained in the animal house for many months.

IV. Our colleague, Dr. J. B. Thiersch, received for examination for another purpose a wild black rat captured in one of the suburbs. On section he found that the kidneys contained inclusions identical with those in the opossums; the only difference apparent in the rat was the greater frequency of giant nuclei both with and without inclusions. Of 16 wild rats, black or brown, trapped subsequently in the city or in our own wood-pile, 3 showed similar inclusions. Six laboratory rats kindly supplied by Mr. H. R. Marston of the Nutrition Laboratory, University of Adelaide, and kept previously under conditions approximately similar to those obtaining in our animal-house, harboured no inclusions. A few wild mice caught in the Institute showed no inclusions.

Our animal-house was built to be rat-proof, and we believe it to be so. However, we could not exclude possible contamination, prior to receipt, of foodstuffs and bedding with a pathogenic agent excreted by rats.

In a further experiment, therefore, we kept unilaterally nephrectomized opossums in cages with a bottom of wire-mesh and containing no bedding. As an additional precaution to prevent possible access of rats and mice we constructed a special cage-rack designed to this end. Five opossums were fed daily with 3 oz. of an autoclaved mixture of equal parts of rolled oats, pollard, crushed maize and minced meat, a half-apple autoclaved, a washed banana peeled at the time of feeding, lemon juice squeezed from washed lemons, and a tablespoon of washed brewer's yeast collected under conditions eliminating contact with rats. We placed the food in recently sterilized dishes and the opossums ate it within a few minutes. Six control animals received the same diet unautoclaved. All the control animals showed numerous inclusions after 72–102 days. Three of 5 opossums on the autoclaved diet showed fewer and earlier inclusions after 81–101 days, while the two others showed none after 55 and 85 days respectively. It will be remembered that in previous dietetic experiments all the animals did not develop inclusions.

Other Inclusions Observed in the Absence of a Known Virus Disease.

In our experimental work to date we have examined more or less comprehensively the organs of over 1,000 monkeys, nearly 4,000 rabbits, 500 guinea-pigs, several thousand mice, nearly 200 sheep, about 50 dogs, about 60 ferrets, and unknown, but relatively small, numbers of pigs, horses, cattle and domestic fowls. We are unable to say in what proportion we have examined the kidneys by means suitable for detecting inclusions, but the numbers are large. We have observed inclusions not due to the experimental procedure employed only in the following.

Monkey (M. rhesus). The kidney showed acidophilic intranuclear inclusions of herpetic type lying in the cells of the ducts of Bellini. Other greatly enlarged nuclei contained no inclusions but exhibited a general excess of chromatin often condensed on the nuclear membrane.

Rabbit. The external secretory cells of the pancreas contained minute acidophilic spherules numbering one to five in each nucleus affected. Rarely they appeared "hollow", as is often the case in the inclusions of poliomyelitis and equine encephalomyelitis. In many cells the nucleolus persisted. Some of the nuclei were slightly enlarged. Occasionally, similar bodies appeared, as if extruded, in the cytoplasm. The islets of Langerhans were unaffected.

Rabbit. Acidophilic nuclear inclusions of herpetic type were numerous in the cells lining the pelvis of the kidney.

Calf. The intranuclear Smith inclusions (see above) in the convoluted and Henle tubules of the kidney are more irregular in shape than those described in this paper. In unstained preparations they appear "crystalline" and "disc-like". They may be multiple in a single nucleus and are not enveloped in basichromatin, nor is the chromatin of the nucleus appreciably affected. They are (in our sections) always acidophilic. The nucleolus is preserved.

Our inclusions did not resemble those produced by physico-chemical means by Davenport *et al.* (1931), Lee (1936), Olitsky and Harford (1937), and others.

COMMENT.

The problem of intranuclear inclusions has been reviewed fully by Cowdry (1934, 1937, 1940) and there would be no point in repeating what he has written.

SUMMARY.

Intranuclear inclusions appeared in the kidneys of Australian opossums kept under hygienic laboratory conditions. In their earlier stages they resembled those in many diseases due to viruses. Their later forms were of bizarre appearance and unlike anything we have seen previously. They were not present in the kidneys (obtained by unilateral nephrectomy) of over sixty opossums recently captured. In a great majority of animals, they developed after a minimum sojourn in the animal house of approximately three weeks. Similar inclusions were present at the time of capture in the kidneys of four of seventeen wild rats (black and brown). None developed in many months in our stock rabbits, guinea-pigs or mice, or in the stock rats of another laboratory. We believe our animal house to be rat-proof; attempts to protect the animals against an infective agent introduced with food or bedding were unsuccessful. Various diets tried did not clearly reduce the frequency of development of these bodies.

REFERENCES.

Cowdry, E. V. (1934): Arch. Path., 18, p. 527.
Cowdry, E. V. (1937): Scient. Monthly, 45, p. 266.
Cowdry, E. V. (1940): Amer. J. clin. Path., 10, p. 133.
Davenport, H. A., Ranson, S. W. and Terwilliger, E. H. (1931): Anat. Rec., 48, p. 251.
Hurst, E. W. (1937): Brit. J. exp. Path., 18, p. 205.
Lee, J. (1936): Amer. J Path., 12, p. 217.
Olitsky, P. K. and Harford, C. G. (1937): Amer. J. Path., 13, p. 729.
Smith, T. (1930): J. exp. Med., 51, p. 519.

EXPLANATION OF PLATE.

Fig. 1. Opossum. Acidophilic inclusions in the nuclei of the convoluted tubules. In some cells a nucleolus is clearly visible. The arrow points to a necrotic cell. Phloxine-methylene blue.

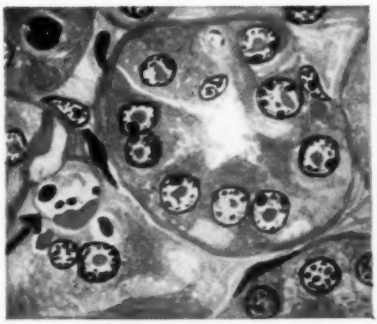
Fig. 2. Opossum. Acidophilic nuclear inclusions surrounded by a clear space and associated with a thickened nuclear membrane as in many virus diseases. Alum-haematoxylin and eosin.

Fig. 3. Opossum. Basophilic inclusions. Phloxine-methylene blue.

Fig. 4. Opossum. Strongly eosinophilic inclusions with a more deeply coloured rim. Methyl blue-eosin (Mann's stain).

Fig. 5. Wild rat. A greatly hypertrophied nucleus with an acidophilic inclusion of irregular shape. The inclusion is surrounded by a thin "capsule" of nuclear chromatin. Iron alum-haematoxylin and eosin.

Fig. 6. Opossum. Composite drawing depicting (left) a cell undergoing mitosis next to one showing in addition to a nucleolus two acidophilic inclusions of irregular shape: (top right) a greatly enlarged nucleus showing an inclusion with basophilic core and weakly acidophilic periphery; note the relation of the inclusion to the general nuclear chromatin more or less radially arranged: (lower right) an inclusion with a smaller basophilic core and definitely acidophilic covering on which the nuclear chromatin is plastered. Phloxine-methylene blue.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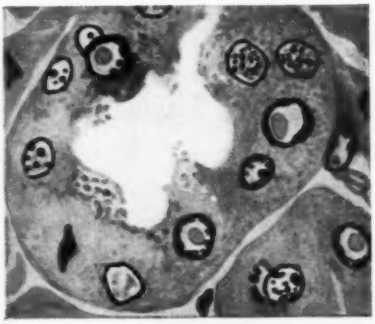
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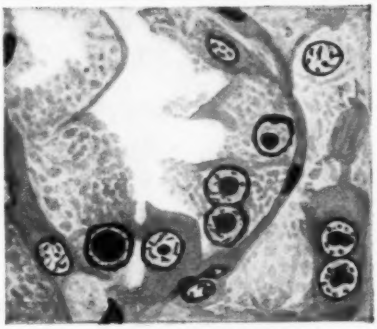
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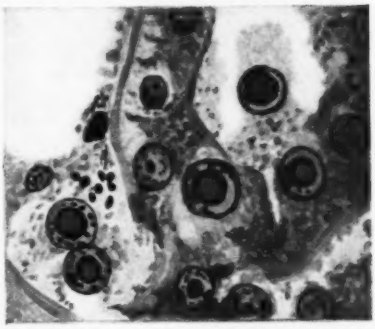
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THE EFFECT OF SALICYLATES (A) ON THE ESTIMATION OF THIAMINE BY THE THIOCHROME METHOD, (B) ON THE EXCRETION OF THIAMINE

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(Accepted for publication 23rd June, 1943.)

A. THE EFFECT OF SALICYLATES ON THE ESTIMATION OF THIAMINE BY THE THIOCHROME METHOD.

Using the thiochrome method of Wang and Harris (1939) for estimating the thiamine content of 24-hour specimens of urine from hospital patients, it was found that when salicylates were present the isobutanol extracts gave a blue fluorescence which resembled and masked that given by thiochrome itself; in some cases the blank showed greater fluorescence than the unknown. The taking of a single aspirin tablet during the test was sufficient to make the titrations difficult or impossible to read accurately. This interference by salicylates has not been recorded in the literature. If, however, the urine was made markedly acid with concentrated HCl almost all the salicylates could be removed by a preliminary extraction with isobutanol. The titrations could then be carried out satisfactorily and the Gerhardt test was found to be negative or faintly positive. When larger quantities of salicylates were present (giving a purple colour with ferric chloride) it was sometimes necessary to extract the acidified urine twice or even three times with isobutanol. Urines which gave a marked positive test with ferric chloride in dilutions of 1 in 10 required still more extractions, and recovery of added thiamine was poor. As Wang and Harris (1939) recommend a preliminary extraction of the urine with isobutanol to remove other fluorescent compounds, this method of removing salicylates takes little extra time, and is satisfactory for all except urines containing very large amounts of salicylates.

The recovery of added thiamine from urine containing salicylates and treated with acid was much greater than from the unacidified urine (Table 1), and except with very large amounts of salicylates was almost as great as that from normal urine or normal acidified urine (Table 2).

Method.

Four estimations were carried out on each specimen of urine using the following dilutions: (1) 6 c.c. urine + 2 c.c. water. (2) 6 c.c. urine + 1 c.c. water + 1 c.c. concentrated HCl. (3) 6 c.c. urine + 1 c.c. water + 1 c.c. pure thiamine solution (containing 1 or 2 γ thiamine). (4) 6 c.c. water + 1 c.c. concentrated HCl + 1 c.c. pure thiamine solution. Each urine was then given one or more preliminary extractions with 5 c.c. of isobutanol and the thiamine determined by the method of Wang and Harris (1939).

Results.

TABLE 1.

Average of thiamine estimations on 22 urines containing salicylates.

	Before addition of acid.	After addition of acid.
Average thiamine in 8 c.c. urine (γ)	0·2 (0 to 1·6)	0·54 (0 to 1·5)
Average thiamine in 8 c.c. urine after addition of 2 γ thiamine (γ)	0·78 (0 to 2)	1·76 (1·3 to 2·6)
Average p.c. recovery	30 (0 to 60)	62 (43 to 90)
Gerhardt test	+ to +++	− to ±

TABLE 2.

Average recovery of thiamine from normal urine.

	On normal urine.		After addition of acid.	
No. of samples.	Thiamine added per sample (γ).	Average p.c. recovery.	Thiamine added per sample (γ).	Average p.c. recovery.
7	1	77	1	81
4	2	63	2	63

Better recovery of added thiamine has been obtained by using relatively larger quantities of methanol. Harris and Wang (1941) found that only 50–70 p.c. of the true value for thiamine was obtained when methanol comprised less than 50 p.c. total volume before the addition of the sodium hydroxide. In the experiments already described the methanol amounted to only 33–44 p.c. of this volume. When the volume of methanol was increased the results shown in Table 3 were obtained.

TABLE 3.

Influence of volume of methanol on the recovery of added thiamine from urine.

	No. of samples.	Thiamine (γ) added per sample.	Salicylates (before extraction).	Average p.c. recovery of added thiamine. Methanol $>$ 50 c.c. volume.	Methanol $<$ 50 p.c. volume.
Without acid	3	2	—	90	69
	3	1	—	94	80
	7	0·4	—	99	89
	3	0·4	++ to ++++	0*	0*
	3	1	++ to ++++	0	0
	3	2	++ to ++++	0*	0*
After acid	3	0·4	++++	99	91
	4	1	++++	90	61
	5	2	++++	94	75

* In one case blank tube showed greater fluorescence than unknown.

B. THE EFFECT OF SALICYLATES ON THE URINARY EXCRETION OF THIAMINE.

Samuels *et al.* (1940) reported an increased excretion of ascorbic acid when salicylates were given to guinea-pigs and rats. Daniels and Everson (1936) and Keith and Hickman (1938) noted a similar increase in excretion with children though Youmans (1937) failed to confirm this effect in adults. It has been known for some time that salicylates increase the urinary excretion of total nitrogenous substances and uric acid (Grabfield and Knapp, 1928) and allantoin (Grey and Grabfield, 1934). This section reports the effect of salicylates on the excretion of thiamine.

During the routine examination of the thiamine excreted in 24-hour samples of urine from a number of hospital patients, it was noticed that in five patients low, or relatively low, values were recorded during prolonged therapy with sodium salicylate. On the other hand in four out of six cases relatively high values were obtained after single doses of, or short periods of treatment with, salicylates.

Method.

The thiamine was estimated by the method of Wang and Harris (1939) after the salicylate had been removed by the method already described.

Results.

In Table 4 the thiamine excreted by five cases of rheumatic fever in whom treatment with sodium salicylate had been continued for ten or more days is shown. In all cases low or relatively low values were obtained.

In Fig. 1 the thiamine excreted per day and the effect on thiamine excretion of single doses of salicylates are recorded in five other cases. In three of these an increased excretion after salicylates was noted. The clinical history of cases 7 and 11 was not significant, but case 8 had been taking up to seven A.P.C. tablets a day for several weeks. The last A.P.C. tablet was taken twenty-four hours before the test was started. Both the cases which showed no response had

TABLE 4.

Effect of salicylate therapy on urinary excretion of thiamine.

Case No.	Before salicylate therapy. Average daily excretion of thiamine (γ).	During salicylate therapy. Daily excretion of thiamine (γ).	Number of days since salicylate therapy started.	Na salicylate taken each day (grains).	After salicylate therapy. Daily excretion of thiamine (γ).	Number of days since salicylate last taken.
1	181*	0 and 0	17 and 21	50	—	—
2	124*	0 and 0	11 and 12	50–60	95 and 96	10 and 11
3	129†	105 and 70	32 and 33	90	—	
4	230†	88 and 112	40 and 41	50	—	
5	100*	—	—	30	88 and 86 138	3 and 4 8

* Average from patients in same ward. † Average from same patient before treatment.

recently shown symptoms possibly due to deficiency, the one of thiamine itself and the other of vitamin B complex. The case notes of these two subjects are:

Case 9. Man, aged 32, who for two months had been having a poor diet and intermittent numbness of the 4th and 5th fingers of the left hand. The symptoms disappeared after receiving 4 mg. thiamine hydrochloride t.i.d. for 2 weeks, but reappeared 3 weeks later (about 6 weeks before this estimation of thiamine in the urine).

Case 10. Woman, aged 51, with achlorhydria and migraine. For several years has had recurrent attacks of fibrositis or "neuritis" and cheilosis. Usually has a high carbohydrate diet with fruit and vegetables but little meat and few eggs. Has occasionally taken supplements of vitamin B complex (Combex) with general improvement in health.

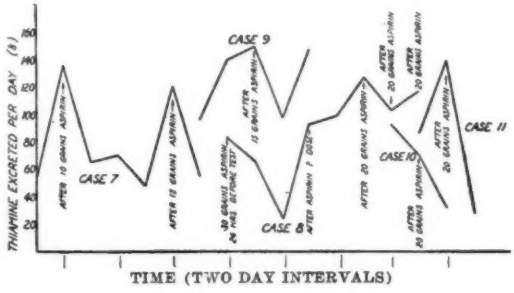

Fig. 1. Effect of single doses of salicylates on excretion of thiamine. (In each case aspirin was taken during the first twelve hours of the twenty-four hour collection period.) For particulars of cases see text.

Another case (Case 6 not shown in Fig. 1) received 50 grains aspirin twenty-four hours before the test was started, but none during the test. The average thiamine excreted per day for the next two days was 94 micrograms. He then received 50 grains sodium salicylate per day for five days. During the 5th day 180 micrograms thiamine were excreted.

Experiments were then carried out to see whether the low values obtained after salicylate therapy, or the high values obtained after single doses could be attributed solely to the salicylates.

The thiamine excreted in the urine each day by 9 subjects (five normal persons and four hospital patients with no evidence or likelihood of thiamine deficiency) was measured over two periods of five consecutive days. At the beginning of the third day of each period the subject received an intramuscular injection of either 1 or 2 mg. thiamine. The second five-day period was started on the same day of the week as the first period (i.e. two days after the completion of the first period) so that by arrangement the menu offered on corresponding days of each period was practically the same. During one of the five-day periods the subject received 10 to 30 grains of aspirin or sodium salicylate each day, the first dose being taken at the beginning of the test.

TABLE 5.

Thiamine excretion per day for five days with and without taking salicylates.

Subject.	Salicylates taken during first 5-day period.						Salicylates taken during second 5-day period.											
	I		II		III		IV		V		VI		VII		VIII		IX	
Particulars.	M. age 51, R. hemiplegia.		F. age 26, normal.		F. age 25, normal.		F. age 12, Psoriasis.		F. age 37, T.B. abscess.		F. age 17, T.B. lesion of skin.		M. age 20, normal.		F. age 29, normal.		F. age 62, normal.	
Salicylate (s) / Aspirin (a) } grains taken per day.	45(s) —		20(s) —		10(a) —		—	30(s)	—	30(s)	—	30(s)	—	20(a)	—	10(s)	—	15(s)
Thiamine excreted per day (micrograms) 1st day	174	115	141	104	141	105	160	106	56	158	65	92	199	280	114	171	—	—
2nd day	150	98	136	45	154	90	85	115	92	170	183	100	81	141	119	102	194	170
3rd day*	630	236	231	120	221	182	154	180	340	350	138	540	300	480	115	441	330	414
4th day	150	118	216	93	178	137	72	163	186	190	256	154	196	265	138	113	220	288
5th day	112	108	201	98	128	90	74	241	150	306	42	84	146	238	200	113	156	342
*Thiamine injected on 3rd day (mg.)	2	2	1	1	1	1	2	2	2	2	1	1	2	2	2	2	2	2
Ratio of thiamine excreted in 5 days with salicylate: Thiamine excreted in 5 days without salicylate. as p.c.	180		201		136		146		143		142		152		137		135	

In Table 5 the thiamine excreted each day in each of the nine test cases is recorded. These results have been examined statistically; in each case there was a significant rise in the thiamine excreted while salicylates were being taken. (Mean daily excretion of thiamine without salicylates 144 γ; with salicylates 216 γ. Standard error $\pm$ 10.) This increase was of about the same order as the increased excretion of uric acid and total nitrogen (Grabfield and Knapp, 1928), and of ascorbic acid (Daniels and Everson, 1936; Keith and Hickman, 1938) found under similar circumstances; but it could not be correlated with variations in the volume of urine excreted.

As approximately the same results were obtained with or without taking into account the increased excretion due to the injection, the salicylates must affect the excretion rather than the absorption of the vitamin. In two cases (subjects 6 and 8), however, the total increased excretion of thiamine with salicylates could be accounted for by the increase occurring in the 24 hours following the injection of thiamine; but in both these subjects there was little or no immediate response to the injection under normal conditions.

DISCUSSION.

Although the number of cases is insufficient to warrant wide generalizations, the results shown in Table 5 and Fig. 1 indicate that salicylates may have a marked effect in increasing the urinary excretion of thiamine over short periods. This increased excretion is not the result of increased absorption.

In some cases (Case 5, Table 4, Case 6; Case 8, Fig. 1) the withdrawal of salicylates was followed by a reduced thiamine output for a few days, presumably until the depleted body stores were replenished. The large differences between the values obtained with and without salicylate treatment in subjects 1 and 2 (Table 5) are also probably due to this effect, as in both these cases the period with salicylates preceded the normal period. The only two cases in which no appreciable rise in thiamine excretion occurred immediately after taking salicylates (Cases 9 and 10, Fig. 1) had both recently shown symptoms attributable to vitamin B deficiency; it seems reasonable to suggest that in these cases the tissue stores had already been depleted or were incapable of being mobilized.

Samuels, Ritz *et al.* (1940) found in rats, following the withdrawal of salicylates, a similar fall in the excretion of ascorbic acid below the original control level. They also observed that guinea-pigs receiving less than a certain quantity of ascorbic acid in their food failed to excrete larger quantities of ascorbic acid when salicylates were given.

The amount of thiamine excreted in the urine in 24 hours depends therefore not only on the thiamine status of the individual at the time, but also on whether salicylates have been taken just prior to or during the test. Variations in thiamine excretion due to salicylate administration will not alter the clinical interpretation of the results if the thiamine status of the patient is very low or very high, but with borderline cases it may have a marked effect. Many people take aspirin or A.P.C. tablets frequently and, in particular, for conditions such as "neuritis" where an estimation of the thiamine status might be of use to clinicians. It is possible that some of the inconsistencies observed in this method of evaluating the status may be due to the effect of salicylates. More constant agreement has been obtained between the quantities of thiamine excreted in two consecutive 24-hour specimens of urine since patients have been asked to refrain from taking salicylates for several days prior to and during the collection of the samples. (When necessary some other analgesic was supplied.)

Longer periods of treatment with large doses of salicylates (as in rheumatic fever) appear to lead to a reduced thiamine output, presumably due to a loss of body stores during the preliminary period of increased excretion (see Table 4). It is, however, a little difficult to reconcile these low figures with the absence of any symptoms which could be attributed to thiamine deficiency. No records of thiamine deficiency associated with rheumatic fever have been found, though Rinehart (1935) has recorded a number of cases of scurvy associated with rheumatoid arthritis treated with salicylates. Possibly the symptoms of salicylate poisoning develop more rapidly than do those of thiamine deficiency, so that salicylate treatment is stopped before the latter are recognized. It is also possible that the low thiamine excretion is due to a raised renal threshold, and that the mobilization of the body stores of thiamine is partially responsible for the action of salicylates in

relieving pain. It seems reasonable to suggest that care should be taken to ensure that patients undergoing salicylate therapy should have an extra supplement of thiamine.

SUMMARY.

Salicylates give a blue fluorescence in ultraviolet light and therefore interfere with the thiochrome method of estimating thiamine. They are not removed by the usual preliminary extraction with isobutanol unless the solution has been made markedly acid.

Over short periods salicylates increase the excretion of thiamine in urine, but long periods of therapy cause a reduced output. The significance of these results on the interpretation of the thiamine excretion test is discussed. It is suggested that a high thiamine intake is advisable during salicylate therapy.

REFERENCES.

Daniels, A. L. and Everson, G. J. (1936): Proc. Soc. exp. Biol. and Med., 35, p. 20.
Grabfield, G. P. and Knapp, E. (1928): J. Pharmac. and exp. Therapy, 32, p. 341.
Grey, M. G. and Grabfield, G. P. (1934): *Ibid.*, 52, p. 383.
Harris, L. J. and Wang, Y. L. (1941): Biochem. J., 35, p. 1050.
Keith, J. D. and Hickman, E. M. (1938): Arch. Dis. Childhood, 13, p. 125.
Rinehart, —. (1935): Ann. int. Med., 9, pp. 586 and 671 (as quoted by Daniels and Everson, 1936).
Samuels, L. T., Ritz, N. D. and Poyet, E. B. (1940): J. Pharmac. and exp. Therapy, 68, p. 465.
Wang, Y. L. and Harris, L. J. (1939): Biochem. J., 33, p. 1356.
Youmans, J. B., Corlette, M. B., Frank, H. and Corlette, M. (1937): Proc. Soc. exp. Biol. and Med., 36, p. 73.

THE BLOOD PIGMENT AND CYANIDES; A GREEN HAEMOGLOBIN[1]

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(Accepted for publication 16th July, 1943.)

The relations between cyanide and the blood pigment have for some years been somewhat confused. While some workers, e.g. Yoshizawa (1937) or Stitt and Coryell (1939), professed to prepare a compound of hydrogen cyanide and reduced haemoglobin analogous to those of haemoglobin with carbon monoxide or with oxygen, other workers, e.g. Brooks (1939), Haurowitz (1925) were unable to find any evidence for such a compound. The compound of cyanide with methaemoglobin, variously known as cyanomethaemoglobin or ferrihaemoglobin cyanide, has of course long been known (see Haldane, 1899) though occasionally, e.g. Schmidt (1938), Drabkin and Austin (1939), it has been erroneously designated cyanhaemoglobin. Its precise constitution has perhaps not been firmly established. By the use of their term "ferrihaemoglobin cyanide" Coryell, Stitt and Pauling (1937) would appear to imply something in the nature of an unionized salt or ester.

As described by Balthazar and Philippe (1926) when cyanomethaemoglobin, in presence of a little excess cyanide is reduced with sodium hyposulphite in the presence of ammonia or sodium carbonate (i.e. in weakly alkaline solution), a brilliant but transient two-banded spectrum is seen which fades according to circumstances, rapidly or slowly to a spectrum with a single diffuse band. Stitt and Coryell (1939) declared that in the presence of a great excess of hydrogen cyanide this two-banded spectrum does not fade and is in fact the spectrum of a diamagnetic cyanhaemoglobin corresponding in constitution to carbon monoxide haemoglobin, having no unpaired electrons around the iron atom. From their account, it was necessary to extrapolate their readings back to zero time to compensate for a steady fall in their susceptibility values. Some of their solutions became gels during the determinations of susceptibility. As will be seen later certain reactions among the blood-pigment proteins have no effect on susceptibility determinations though of importance in connection with their constitution. Again, the gradual rise of the single broad band in the spectrum so softens the initial brilliant two-banded spectrum as to cause the bands of the latter to resemble those of, say, carbon monoxide haemoglobin much more than they do when freshly formed, especially to workers not sufficiently accustomed to disappointment in visual spectroscopy.

Another aspect of this matter is the work of Barkan and Schales (1938) who showed that, in the presence of a little potassium cyanide, hydrogen peroxide rapidly destroys oxyhaemoglobin which in the absence of the former is almost indifferent to the latter reagent. The product of the reaction after reduction is, according to Lemberg (1941), essentially denatured globin cyancholehaemochromogen together with other products in lesser yield. The porphyrin ring is split and the globin denatured.

Yet another aspect is the observation made in the course of experiments described in this paper that ascorbic acid, in the presence of a little cyanide at pH 8–9

[1] This work was aided by a grant from the Australian National Health and Medical Research Council.

rapidly converts oxyhaemoglobin, not to choleglobin as in Lemberg's (1941) experiments without cyanide, but to cyanomethaemoglobin. Coupled with Schmidt's (1938) demonstration of the difficulty of reducing cyanomethaemoglobin compared with that of methaemoglobin this supports Lemberg's view that methaemoglobin enters the cycle of choleglobin formation. Normally its concentration in this cycle is insufficient for its spectrum to be observed.

The experiments in this paper were undertaken in the hope of throwing some light on the nature of the transitory substance with the two-banded spectrum. They perhaps shed some light on other matters as well.

EXPERIMENTAL.

So many workers appear, either like Barkan and Schales (1938) to forget that potassium cyanide is a caustic alkali, or else to make clumsy efforts to circumvent, or allow for, its effects that it must be clearly understood that throughout this work, unless specially indicated, the cyanide was always used in the form of potassium cyanide together with an equal weight of ammonium chloride. When for any reason they were added separately the latter was always added to the protein first and well mixed. The cyanide was always added slowly with adequate agitation to prevent any dangerous local degree of alkalinity. Other, nitrogen-free, buffers were tried but since they neither caused any difference nor offered any advantage (rather the reverse) they were discarded.

Cyanomethaemoglobin differs from many other haemoglobin complexes in its stability. The cyanide cannot be removed by physical means, dialysis, precipitation or crystallization, but only by chemical action, e.g. reduction of the iron atom. If cyanomethaemoglobin with little or no excess cyanide in the solution is reduced with sodium hyposulphite near the neutral point the two-banded spectrum first seen rapidly gives place to a single-banded spectrum which is that of haemoglobin; this can be converted to carbon monoxide haemoglobin with coal gas. At pH 6·5–7, to avoid the deleterious effects on the pigment of the oxidation of the reducer, the carbon monoxide can be readily replaced by gaseous oxygen. This has been observed too often by too many workers to be doubted. It was carefully confirmed during these experiments using a reversion spectroscope to measure the positions of the bands. The oxyhaemoglobin was converted to haemoglobin in a vacuum and restored to oxyhaemoglobin by aeration. With higher concentrations of cyanide, at the same pH, the spectral change was slower and it was possible to observe the bands more attentively and measure their positions. This observation revealed the spectrum as having, when fresh, much more the appearance of a haemochromogen than of a haemoglobin-gas compound. This applies not only to the wavelengths of the bands but to their appearance and intensity and to the brilliance of the whole spectrum. In fact it must be regarded as a superb specimen of this class. When the reduced solution was aerated cyanomethaemoglobin and some oxyhaemoglobin were visible.

Some experiments on the formation of "cyanhaemoglobin" were also carried out in a small glass apparatus from which all the oxygen was removed before the solutions were mixed. Of the substances tried cyanomethaemoglobin showed on reduction the most brilliant two-banded spectrum which, within a few minutes in the absence of oxygen, changed to that of haemoglobin. When haemoglobin, free from both oxyhaemoglobin and methaemoglobin was treated with cyanide in the absence of oxygen, at about pH 9 to avoid denaturation, no two-banded spectrum was seen unless the cyanide concentration was about normal, when a faint band at 569 mμ slowly formed above the band of haemoglobin. On the admission of oxygen to this solution it turned steadily dark green and within a few minutes most of the pigment appeared to have changed to green haemoglobin leaving but little in the form of any protohaemoglobin derivative. The great readiness with which the two-banded substance was obtained from cyanomethaemoglobin shows how more nearly it is related to it than to the oxyhaemoglobin group of compounds.

On a second reduction with sodium hyposulphite a small fine band in the red portion of the spectrum appeared which faded on aeration. Successive reductions and aerations augmented this band and diminished those of oxyhaemoglobin and cyanomethaemaglobin till they became invisible. After several reductions a little denatured globin protohaemochromogen was seen. Parallel experiments showed that a pH of about 9–10 was suitable and that a high concentration of cyanide was helpful. In spite of the rather drastic treatment, the protein, now of a bright dark green colour, remained mostly soluble in neutral solution. An experiment on a rather larger scale showed it to be soluble in half saturated ammonium sulphate solution but to be precipitated by the further addition of solid ammonium sulphate. The precipitate was filtered off and readily dissolved in a little water. In fact it still showed the general behaviour of a native protein.

For a study of its properties a larger quantity was prepared as follows: To 20 c.c. of fresh well-washed horse erythrocytes in a 1,200 c.c. bottle were added 20 c.c. H_2O, then 10 gm. solid NH_4Cl, then 10 gm. commercial KCN freshly dissolved in 40 c.c. H_2O. The bottle was rotated

slowly about its axis which was horizontal. Through a loosely fitting rubber tube in the neck a slow stream of oxygen was passed from a cylinder. Solid $Na_2S_2O_4$ was added in a fine spray from time to time in about 0·1 gm. lots as soon as the spectrum showed that the pigment was oxidized. The experiment was carried out at about 16° C.

In the presence of unoxidized sodium hyposulphite the bands seen were at first those of the two-banded haemochromogen-type spectrum. Less haemoglobin was seen than in the experiments in the enclosed apparatus. Soon the band in the red became visible gradually increasing in intensity. After a while the bands of oxyhaemoglobin and of cyanomethaemoglobin were no longer visible on oxidation. Three more reductions and oxidations were then effected; 30 c.c. of saturated ammonium sulphate solution were added. This, with the salt already present, precipitated the stroma and the denatured protein. This was filtered off at 0° C. The precipitate was washed with a little half-saturated ammonium sulphate solution. The protein in the united filtrate and washings was precipitated by the cautious addition of solid ammonium sulphate. The dark green precipitate of protein was filtered off rapidly on a Buchner funnel, washed with a little cold saturated ammonium sulphate solution and redissolved in the smallest possible amount of cold water. The preparation was repeated and the united solutions dialysed at 0° C. against water containing a few drops of 10 p.c. acetic acid per litre. After a few days the dark olive-green solution had a pH of 7·3 and was ready for optical studies. The protein concentration was determined by heat coagulation after the addition of a little formalin and weighing the dried precipitate.

The positions of its bands were measured with a reversion spectroscope; coal gas had no effect on the spectrum of the oxidized form. Attempts to prepare a reversible oxygen compound analogous to oxyhaemoglobin were unsuccessful. The wavelengths of its bands and its general appearance resemble those described for choleglobin (Lemberg, 1941). During the preparation of its substance no trace was seen, however, of any band in the far red (655 mμ) which is so important a characteristic of choleglobin. Unlike choleglobin (Lemberg, 1941) this substance has no power to add on oxygen which the former appears to retain at least in the earlier stages of its preparation. Again (v.i.) the ultra-violet spectrum of this green haemoglobin has a definite Soret band which choleglobin apparently has not. Whether the differences noted should be regarded as entitling this haemoglobin to be considered as a separate entity, may perhaps fittingly await expert judgment. For the present it is proposed to call it "cruoralbin".

TABLE 1.

Maximum absorption in the visible spectrum.

Parent Substance.	Derivative.	Reversion Spectroscope. mμ	Spectrophotometer. mμ	$\epsilon 10^{-3}$	
Haemoglobin	Alkaline methaemoglobin	—	575, 540	9·0–9·2	9·4–10·3
	Denatured globin parahaematin	—	575, 540	8·8–9·2	10·2–10·4
	Native globin haemochromogen	569, 537	—	—	—
	Denatured globin haemochromogen	559, 528	—	—	—
Choleglobin	"CN met."	680	—	—	—
	Reduced	630	—	—	—
	CO	630	—	—	—
	CO haemochromogen	628	630	16·1	—
	Haemochromogen	618–620	618–622	13·2	—
Green haemoglobin	Oxidized	600 (varied with concn.)	610, 590	11·1	11·1
	Reduced	624	—	—	—
	CO	625	620	20·5	—
	Haemochromogen	628	—	—	—
	CO haemochromogen	628	—	—	—
	Treated with NaOH (oxidized)	642	—	—	—

Sources: The methaemoglobin and parahaematin figures are from Drabkin and Austin (1936).
The choleglobin figures are from Lemberg (1941).
The remaining data were determined on the reversion spectroscope used for this paper.
The value for the oxidized form of the green haemoglobin is very uncertain and variable, depending on the concentration. The band, as can be seen from graph 1, is very faint.

The visible absorption curve was measured with a Hilger-Nutting spectrophotometer at the Commonwealth Serum Laboratories by Dr. W. W. Hurst. As can be seen from Fig. 1 the intensity of the band of the carbon monoxide compound is rather striking.

The ultra-violet spectrum was determined with a Hilger échélon cell spectrophotometer. As

can be seen (Fig. 2) there is a small Soret band, rather short and broad, which is unaltered by changing from acid to alkaline solution and but little by reduction in the presence of carbon monoxide. Thus no large proportion of the absorption here can be due to protohaematin derivatives. The effect was tried of the addition of this green haemoglobin to haematin on the ultra-violet spectrum of the latter. Determinations were made with the green haemoglobin in both cells thus cancelling its own absorption and limiting the observed spectrum to that due to the effect of the protein on the haematin. When haematin was in the oxidized state little change was observed from its usual spectrum. On reduction, however, in the presence of carbon monoxide a Soret band

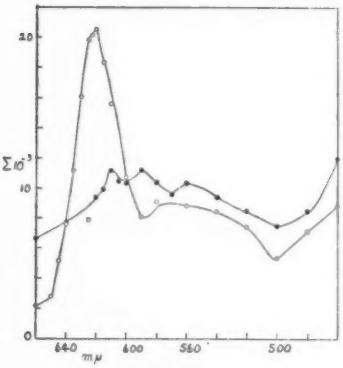

Fig. 1.

—o—o— Green haemoglobin reduced in coal gas.

—●—●— Green haemoglobin as prepared.

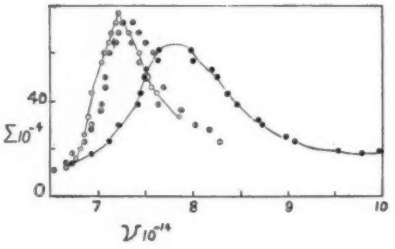

Fig. 2.

—o—o— Green haemoglobin reduced in coal gas.

—●—●— Green haemoglobin as HCl.

ϴ ϴ ϴ Green haemoglobin as prepared, pH 4·7.

Φ Φ Φ Green haemoglobin as prepared, pH 10·6.

Fig. 1. Abscissae: wavelengths in mμ. Ordinates: $\epsilon 10^{-3}$.

Figs. 2–5. Abscissae: $\nu 10^{-14}$ (frequencies) Ordinates: $\epsilon 10^{-4}$.

$$\epsilon = \frac{M}{cd} \log \frac{I_0}{I}$$

where M = equivalent weight. c = concentration in grams per litre. d = depth in centimetres. I_0 = intensity of entering beam. I = intensity of emergent beam.

of great intensity was introduced into the spectrum, equal to the highest observed for protohaematin derivatives. While this intense band was observed only when the protein was present in the proportion of at least one equivalent (16,700 gm.) per gm. molecule of haematin, the Soret band when smaller amounts of green haemoglobin were employed was sufficiently remarkable and higher than the values found for denatured globin protohaemochromogen (Hicks and Holden, 1932) or for its carbon monoxide compound (Holden and Lemberg, 1939). There can be little doubt that, without being "denatured", i.e. converted to a meta-protein insoluble at its iso-electric point, the globin molecule as well as its prosthetic group has undergone a profound change of structure. A similar "un-masking" of haemochromogen-forming groups occurs on the denaturation of globin and other proteins (Holden and Freeman, 1929) but it must now be regarded as incidental rather than as essential to denaturation.

It was not found possible to remove with acid any significant proportion of the prosthetic group. The protein is readily denatured by the usual agents. The spectrum of the reduced form shows little change on the addition of much caustic alkali, whether carbon monoxide be present or not.

The effect of change of pH on the ultra-violet spectra of methaemoglobin and of cyanomethaemoglobin was verified since it is not easy to find accurate figures in the literature. The effect of cyanide on the ultra-violet spectrum of haemoglobin was also determined and the data are included. The slightly higher figure obtained for alkaline methaemoglobin than that found by Hicks and Holden (1929) has been retained as more probable.

DISCUSSION.

There would appear to be four problems towards the solution of which some advance may have been made in this paper: the nature of the transitory substance whose spectrum has two bands in the green region of the spectrum; the single-banded substance into which it is spontaneously transformed; the nature of the green haemoglobin and the steps in its production; the part played by hydrogen cyanide in these affairs.

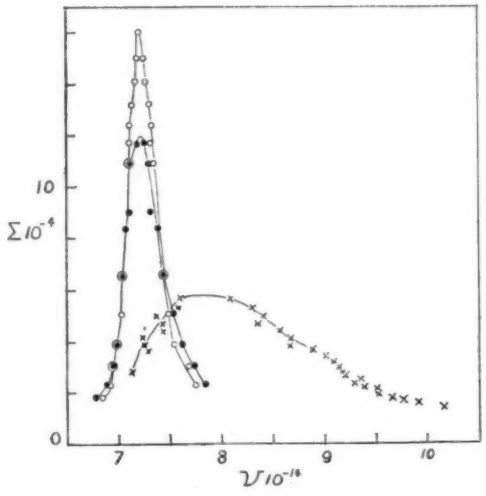

Fig. 3.

—o—o— Green haemoglobin CO protohaemochromogen, 1·1 equivalent of protein per equivalent of haematin.

—•—•— Green haemoglobin CO protohaemochromogen, 0·15 equivalent per equivalent of haematin.

—x—x— Alkaline haematin in the presence of green haemoglobin, pH 10·8.

Any attempt at a discussion of these problems involves first an appraisement of the values to be assigned to each type of physical evidence and a consideration of the structural relations among the blood-pigment derivatives as revealed by that evidence. In this field differences among absorption spectra have been more often used as evidence for the existence of new compounds than close similarities as evidence of related structures. Only in the presence of oxygen does globin show clearly any peculiarity it may confer on reduced haematin. The spectra of the haemochromogens and carbon monoxide haemochromogens of other proteins are almost indistinguishable from those of denatured globin under appropriate conditions. The visible spectrum of carbon monoxide haemoglobin is nearly the same as that of denatured globin carbon monoxide haemochromogen. The visible spectrum of alkaline methaemoglobin resembles those of the parahaematins (''ferrihaemochromogens'', ''oxidized haemochromogens'') of many other proteins and nitrogenous substances. A good example of this can be seen in the work of Drabkin and Austin (1936) where the observed spectra of alkaline methaemoglobin and denatured globin parahaematin are almost exactly identical. While it is true that most parahaematins are formed only in neutral solution those of denatured egg albumin and casein are formed only in solutions more strongly alkaline than is suitable for the formation of alkaline methaemoglobin and are dissociated at the neutral point (Holden and Freeman, 1929). In a very low concentration of alkali and the presence of human serum the visible absorption spectrum of ''neutral haematin'' differs from that of methaemoglobin less than do two preparations of pyridine haemochromogen from each other when made under different conditions of pH and salt concentrations (Anson and Mirsky, 1925). Such variations as these last are not now held to be proof of the existence of different compounds. Drabkin and Austin (1936) found that the visible spectra of cyanhaematin and cyanomethaemoglobin were practically identical. In fact the best evidence for the existence of globin-pigment compounds as a select group may lie in many cases with the results of their discreet chemical disintegration rather than with physical measurements.

The Soret band in the ultra-violet region must be regarded primarily as evidence of a more or less intact porphyrin ring and of the distribution of the pigment molecules singly outside, or nearly outside, each other's radius of action, at least as far as optics are concerned. The small Soret band often observed in the spectra of haematin or porphyrins in the presence of proteins or other nitrogenous substances indicates either a little of the pigment molecules so distributed

or a less complete isolation of the majority. While some form of combination is thus implied it is doubtful whether in many cases it would be otherwise discernible. It may be worth recording that the author has prepared, in small amount, breakdown products of haematin by repeated oxidation and reduction of such haematin-protein complexes. He has observed marked decreases in the height of the Soret band of acid haematin in organic solvents to be due to an increase in the concentration used for the determination. This can be attributed only to a partial aggregation of the molecules of the pigment. The visible spectrum of protoporphyrin in sodium hydroxide solution provides a similar example of spectral variability.

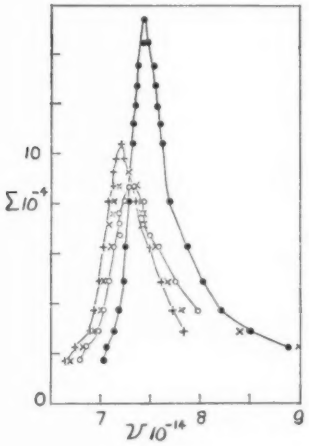

Fig. 4.

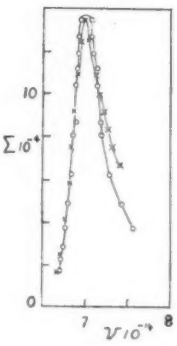

Fig. 5.

Fig. 4. —o—o— Methaemoglobin pH 4·68.
—•—•— Methaemoglobin pH 10·8.
—+—+— Cyanomethaemoglobin pH 4·77.
—x—x— Cyanomethaemoglobin pH 10·8.

Fig. 5. —o—o— Haemoglobin in cyanide solution.
—x—x— Haemoglobin in water.

In assessing the value of magnetic data it must be remembered that few derivatives of protohaemin have susceptibilities in exact agreement with the theoretical predictions of this precise method. The "correct" value for haematin was obtained by Pauling and Coryell (1936) only by dissolving it in a 30 p.c. aqueous sucrose solution which was certainly empirical and, indeed, somewhat unnatural. The matter was investigated by Rawlinson (1940). Perhaps one of the most important lessons to be drawn from susceptibility determinations on blood-pigment derivatives is that they are not, as is often tacitly assumed, in one set form. Unless there is the strongest evidence to the contrary it would seem within the bounds of reason to accept departures from the expected magnetic values, as from expected spectrometric values, as indications of aggregation or of admixture with other compounds than the one imagined to be present alone.

The great attention attracted by the demonstration of the disappearance of paramagnetism on the conversion of haemoglobin to oxyhaemoglobin has diverted that due to the reactions of the blood pigment which involve less or no change in magnetic susceptibility. Haematin can be converted to methaemoglobin, or haemoglobin to reduced haematin without any change in susceptibility. Whereas the combination of reduced haematin with globin produces no discernible magnetic change its combination with denatured globin does. Methaemoglobin combines with fluoride ion without any change in susceptibility while its compounds with cyanide and with iminazole have different magnetic moments. Alkaline methaemoglobin has a different susceptibility from methaemoglobin. "Cyanhaemoglobin" according to Stitt and Coryell (1939) has the same susceptibility not only as oxyhaemoglobin but also as haemochromogen.

Physical measurements alone can establish the constitution of "cyanhaemoglobin" no more than those of oxyhaemoglobin or methaemoglobin the knowledge of which is due to chemical analyses and observation of their behaviour towards selected reagents. To assign to the substance with a two-banded spectrum, on the basis of a magnetic susceptibility measurement only, a constitution analogous to that of carbon monoxide haemoglobin is to ignore its spectrum and to go against the evidence of its most striking chemical features. Thus it is most readily formed by the reduction of cyanomethaemoglobin in the absence of oxygen, which appears to oxidize it quantitatively as it does reduced haematin. It can not be formed from oxyhaemoglobin by gaseous exchanges, cyanomethaemoglobin resulting from any such attempt. Its reaction with oxygen is entirely different from those of haemoglobin or carbon monoxide haemoglobin. Thus even if one were prepared to disregard its spectrum chemical considerations alone require an entirely different constitution.

TABLE 2.

Maximum absorption in the ultra-violet spectrum.

Substance.	pH.	Source of data.*	Wavelength, mμ.	Frequency, $\nu 10^{-12}$	Molecular extinction coefficient, $\epsilon 10^{-3}$	Graph No.
Methaemoglobin	4·68	2	407	737	154	4
Methaemoglobin	10·8	2, 4	411	730	90	4
Cyanomethaemoglobin	4·77	4	416	721	104	4
„	10·8	4	412	728	92	4
Haemoglobin	—	1	421	713	127	5
„ with cyanide	9·2	4	428	701	135	5
Green haemoglobin	4·7	4	410	732	77	2
„ „	10·6	4	414	725	76	2
Green haemoglobin reduced in CO	9·8	4	416	721	77	2
Green haemoglobin in HCl	1·16	4	384	781	61	2
CO protohaemochromogen of green haemoglobin, 1·1 equiv. of protein per equiv. of haematin	9·8	4	416	721	160	3
CO protohaemochromogen of green haemoglobin, 0·6 equiv. of protein per equiv. of haematin	9·8	4	416	721	155	–
CO protohaemochromogen of green haemoglobin, 0·3 equiv. of protein per equiv.	9·8	4	416	721	124	–
CO protohaemochromogen of green haemoglobin, 0·15 equiv. of protein per equiv.	9·6	4	416	721	120	3
Denatured globin protohaemochromogen	—	1	424	707	110	–
Denatured globin CO protohaemochromogen	—	3	423	709	106	–

* Sources of data for Table 2.

1. Hicks and Holden, 1929, 1932. 2. Holden, 1936. 3. Holden and Lemberg, 1939. 4. This paper.

The work cited of Pauling and his colleagues and that of Barkan and Schales (1938) and of Lemberg (1941) and his colleagues on the oxidative disruption of the blood pigment seem to require a re-examination of the evidence on the union between the prosthetic group and globin and a change from the older idea of a firm link between them as a kind of solid framework for the addition of gases or a change in the valency of the iron atom. To take the spectrometric evidence first: The intense Soret bands of methaemoglobin, carbon monoxide haemoglobin and of the green haemoglobin carbon monoxide proto-haemochromogen described above are of substances in which the conditions of pigment distribution are most perfectly fulfilled. That the protein providing these conditions should be native globin or a close derivative is to be expected. Others of this group are protoporphyrin globin, oxyhaemoglobin and haemoglobin itself. Cyanomethaemoglobin and alkaline methaemoglobin form a second group with lower Soret bands. One is justified in regarding them as substances in which a portion, at least, of the molecules of the pigment are bound to the globin differently from those of the first group. They

differ in susceptibility both from methaemoglobin and from other nitrogenous compounds of methaemoglobin, e.g. iminazole methaemoglobin. In the absence of excess cyanide they pass on reduction rapidly to haemoglobin, i.e. the pigment-protein linkage reverts to the first, the specific type. It may be noted that in general compounds of haematin with nitrogenous substances are more dissociated and less stable than corresponding compounds of reduced haematin. Methaemoglobin is more readily dissociated (to haematin and globin) than haemoglobin by acid or by sodium salicylate (Holden, 1936, 1937).

It is suggested here that alkaline methaemoglobin is in reality (native) globin protoparahaematin mixed with some alkaline haematin colloidally associated with the native globin. On reduction it passes via native globin haemochromogen straight to haemoglobin too fast for the intermediate compound to be visible under the conditions ordinarily used. It may be worth recalling Hill's (1925) work on the effect of sodium hydroxide on haemoglobin at — 5° C. A repetition might show that his explanation of the formation of haemochromogen under those conditions was too modest and that what he had was not globin contaminated by denatured globin haemochromogen but native globin haemochromogen itself. Cyanide forms a dissociable compound with the first group on the globin molecule which reduced haematin can only slowly dissociate. The two-banded spectrum seen during the reduction of cyanomethaemoglobin is that of native globin protohaemochromogen.

It should be clearly realized that this spontaneous conversion of native globin protohaemochromogen to haemoglobin without change of pH is no support for, or connected with, the unverified supposition of Anson and Mirsky (1925) that by a change of pH denatured globin protohaemochromogen, the only one then known, could be transformed into haemoglobin.

Since haemochromogens have the same zero magnetic moment as "cyanhaemoglobin" is supposed to have, magnetic determinations cannot decide the question. There is at present no spectroscopic reason against this view. The striking fact is that, from all spectroscopic appearances, cyanide is totally unable to compete with native globin for the reduced haematin but only with the reduced haematin for the globin. Stitt and Coryell (1939) apparently determined the magnetic susceptibility, not of "cyanhaemoglobin" as they thought, but of native globin haemochromogen. These substances though so different in constitution apparently have, as would be expected from all the analogies, the same magnetic susceptibilities. The drift that they observed is due to the conversion of some of the haemoglobin present to denatured globin haemochromogen. The haemochromogenic groups on the molecule of globin which normally show their presence only in the spectrum of alkaline methaemoglobin (a "parahaematin", "ferrihaemochromogen" or "oxidized haemochromogen") are too near the specific groups for a haematin molecule to occupy each simultaneously. Thus on adding pure reduced haematin in sodium carbonate to pure haemoglobin no haemochromogen spectrum is at first seen.

An essential step in the preparation of green haemoglobin appears to be the oxidation of the (native) globin haemochromogen. Coupled therewith are: (1) The irreversible alteration of the specific groups of globin; (2) The unmasking of the additional haemochromogenic groups of globin; (3) The alteration of the haematin molecules and their recombination with other portions of the globin molecule different from the haemochromogenic groups. This combination cannot apparently be broken down by dilute acids. The fact that all this does not essentially involve denaturation may be placed beside the effects of oxygen on the denaturation of the blood pigment and the oxidizing effect on ascorbic acid of oxygen liberated from oxyhaemoglobin by acid (Lemberg, 1938). Some reagent to combine with the protein seems to be necessary to secure the simultaneous liberation of oxygen from oxyhaemoglobin and the oxidation of the iron atom without denaturing the protein. Examples are potassium ferricyanide and also ferric

sulphate in weakly acid solutions (Hill and Holden, 1927). One is constrained to see, in the protection from denaturation by so drastic a reagent as sodium hyposulphite, evidence for a definite combination of hydrogen cyanide with certain of the groups of the globin that normally suffer from the combined effects of sodium hyposulphite and oxygen in weakly alkaline solution. To a lack of such protection is to be attributed the extensive denaturation occurring during the preparation of choleglobin. Unfortunately, ascorbic acid, though otherwise admirable, appears to be incapable of reducing cyanomethaemoglobin.

Two comments may perhaps be permitted. The intense colour and great intensity of the band of this green haemoglobin suggests that the great discoloration of blood observed in certain diseases may owe something to pigments of this class formed *in vivo* by agents more biological than potassium cyanide.

The inactivation of cytochrome oxidase by cyanide has always been ascribed to a combination of cyanide with its haematin. There seems however a possibility that the point of attack may be rather the specific position in the protein which the reduced form of the haematin would normally occupy.

SUMMARY.

The preparation and some properties of a green derivative of haemoglobin are described.

Its ultra-violet and visible spectrophotometric curves are recorded.

The bearing of this substance on the evidence for the existence of cyanhaemoglobin is discussed.

Acknowledgments. The visible spectrophotometric curves were determined by Dr. W. W. Hurst at the Commonwealth Serum Laboratories, Melbourne. The writer is greatly indebted to the director of the Laboratories and to Dr. Hurst for such generous help. The ultra-violet spectrophotometer is the property of the Australian National Health and Medical Research Council.

REFERENCES.

Anson, M. L. and Mirsky, A. E. (1925) : J. Physiol., 60, p. 50.
Balthazar, V. and Philippe, M. (1926) : Ann. méd. légale, 6, p. 137.
Barkan, G. and Schales, O. (1938) : Z. physiol. Chem., 253, p. 83.
Brooks, M. M. (1939) : Proc. Soc. exp. Biol. and Med., 40, p. 108.
Coryell, C. D., Stitt, F. and Pauling, L. (1937) : J. Amer. chem. Soc., 59, p. 632.
Drabkin, D. L. and Austin, J. H. (1932) : J. biol. Chem., 98, p. 719.
Drabkin, D. L. and Austin, J. H. (1936) : *Ibid.*, 112, pp. 55, 102.
Haldane, J. S. (1899) : J. Physiol., 25, p. 230.
Haurowitz, F. (1935) : Z. physiol. Chem., 232, p. 159.
Haurowitz, F. and Waelsch, H. (1929) : *Ibid.*, 182, p. 82.
Hicks, C. S. and Holden, H. F. (1929) : Austral. J. exp. Biol., 6, p. 175.
Hicks, C. S. and Holden, H. F. (1932) : *Ibid.*, 10, p. 219.
Hill, R. (1925) : Biochem. J., 19, p. 341.
Hill, R. and Holden, H. F. (1927) : *Ibid.*, 21, p. 625.
Holden, H. F. (1936) : Austral. J. exp. Biol., 14, p. 291.
Holden, H. F. (1937) : *Ibid.*, 15, p. 409.
Holden, H. F. and Freeman, M. (1929) : *Ibid.*, 6, p. 79.
Holden, H. F. and Lemberg, R. (1939) : *Ibid.*, 17, p. 133.
Lemberg, R. (1938) : J. and Proc. Roy. Soc. New South Wales, 72, p. 62.
Lemberg, R. *et al.* (1941) : Biochem. J., 35, p. 328 *et seq.*
Pauling, L. and Coryell, C. D. (1936) : Proc. Nat. Acad. Sci. U.S.A., 22, pp. 159, 210.
Rawlinson, W. A. (1940) : Austral. J. exp. Biol., 18, p. 185.
Schmidt, O. (1938) : Biochem. Z., 296, p. 210.
Stitt, F. and Coryell, C. D. (1939) : J. Amer. chem. Soc., 61, p. 1263.
Yoshizawa, S. (1936) : Japan. J. med. Sci., 4, Pharmacol., 10, abstract 4.

APPENDIX.

DETAILS OF SOLUTIONS USED FOR SPECTROPHOTOMETRY.

The stock solution of green haemoglobin contained 23·43 gm. of the protein per litre.

The strength of the stock solution of methaemoglobin was determined spectrophotometrically using 154×10^3 as the molecular absorption coefficient of the head of the ultra-violet band. The absorption coefficients of the substances prepared from this methaemoglobin were calculated from this value.

Fig. 1. Green haemoglobin, 0·8 c.c. stock, 0·1 c.c. $N.NH_3$, H_2O to 25·0 c.c. Green haemoglobin CO, 0·6 c.c. stock, 0·5 c.c. $N.NH_3$ trace of solid $Na_2S_2O_4$, H_2O to 25 c.c. Coal gas passed in; pH 9·0.

Fig. 2. Green haemoglobin, pH 4·7, 0·5 c.c. stock, 1·0 c.c. Walpole's acetate buffer pH 4·63, H_2O to 10 c.c.

Green haemoglobin, pH 10·6, 0·6 c.c. stock, 0·4 c.c. $N.NH_3$, H_2O to 10·0 c.c.

CO compound, 0·5 c.c. stock, 0·5 c.c. $N.NH_3$, 0·4 c.c. of 5 p.c. $Na_2S_2O_4$ in $N.NH_3$, Coal gas, H_2O to 10·0 c.c.

Green haemoglobin in HCl, 0·6 c.c. stock, 1·0 c.c. N. HCl, H_2O to 10 c.c.

Fig. 3. Alkaline haematin spectrum.

Haematin stock is 1·636 mg. haemin, 1·0 c.c. $N.NH_3$, H_2O to 10·0 c.c.

0·4 c.c. green haemoglobin stock, 2·0 c.c. haematin stock, H_2O to 10·0 c.c.

For haemochromogen spectra.

Haematin stock is 3·14 mg. haemin, 0·5 c.c. $N.NH_3$, H_2O to 10 c.c. 0·8 c.c. haematin stock, 0·32 c.c. green haemoglobin stock, 0·4 c.c. 5 p.c. $Na_2S_2O_4$ in $N.NH_3$, coal gas, H_2O to 10·0 c.c. The lower band is a solution of the same composition except that it has only $\frac{1}{3}$ as much green haemoglobin.

Fig. 4. Methaemoglobin derivatives.

Each had 1·2 c.c. of a stock methaemoglobin. The acid ones had 1·0 c.c. of Walpole's acetate buffer pH 4·63. The alkaline ones had 0·5 c.c. of N. ammonia. The ones with cyanide had 0·1 c.c. each of 1 p.c. KCN and 0·1 c.c. each of 1 p.c. NH_4Cl. The total volume was in each case made up to 10·0 c.c. with H_2O.

Fig. 5. Haemoglobin in cyanide.

1·2 c.c. methaemoglobin stock.

8·0 c.c. of 10 p.c. KCN in 10 p.c. NH_4Cl. 0·4 c.c. of 5 p.c. $Na_2S_2O_4$ in H_2O. H_2O to 10·0 c.c. pH 9·2.

The control haemoglobin is from Hicks and Kolden (1929).

A METHOD FOR THE COLORIMETRIC DETERMINATION OF OXYHAEMOGLOBIN

by HENRY FRANCIS HOLDEN

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(Accepted for publication 8th June, 1943.)

In connection with the use of haemoglobin as a reagent for the determination of carbon monoxide in air (Holden, 1943) it was necessary to have a means of carrying out periodical determinations of the amount of horse oxyhaemoglobin present in fresh solutions of this substance. In spite of the vast literature on this subject and the multitude of methods offered there appears as yet no great measure of agreement on this important determination. The recent report to the British Medical Research Council (British Medical Journal, 1943) reviews some aspects of the problem. There is no doubt that in the hands of practised experts the Van Slyke (1924) manometric method gives reliable results but the time required for the preparation of the equipment for occasional determinations and for its subsequent cleansing diverted the writer towards other possibilities. He used at first the Barcroft (1909) differential apparatus and observed the irregularities noted by Haldane (1900) with his ferricyanide method and by Bancroft *et al.* (1928). A series of experiments, however, in which concentrated solutions of horse oxyhaemoglobin were resaturated carefully with oxygen before analysis showed these variations to be due to the initial presence of oxidizable substances which reduced some of the oxyhaemoglobin. Once these had been oxidized consistent results were obtained.

In order to obtain a still simpler method which would yield results reproducible to about 1 p.c. the possibilities of a colorimetric determination were explored. The method adopted is cheap enough to be considered by hospital laboratories as a relief from the acid haematin class of method particularly where it is not desired to provide costly equipment for junior workers.

The difficulty in the colorimetric determination of oxyhaemoglobin arises from its very low absorption in the red region of the spectrum, while the colour of acid haematin is unfortunately unstable. To avoid these difficulties the light used was restricted to that part of the spectrum where the absorption of light by oxyhaemoglobin is much higher. This useful refinement is available in some of the most costly analytical colorimeters in other countries. After a number of trials with such substances as were available it was found that the green glass used in railway signals was suitable as a filter and a piece was inserted in the eyepiece of the colorimeter. A 25 watt opal electric lamp gave light of sufficient intensity. The lamp house, so shaped as to exclude stray light was bolted to the same stand as the colorimeter. As a permanent glass standard with which the various solutions of haemoglobin were to be compared there was chosen a piece of olive-green glass which, strange though it may sound, provided in this blueish-green light an excellent visual match for a solution of oxyhaemoglobin. The blood or concentrated oxyhaemoglobin solution, of which the concentration had been previously determined with the Barcroft apparatus was diluted with 0·005 N ammonia, and the colorimetric determination performed at once.

In order that the measurements of the amounts of the strong haemoglobin solutions used should be of sufficient accuracy for an exact comparison of the two methods, a specially shaped pipette was made whose capacity was accurately determined. It was filled with the oxyhaemoglobin solution, weighed and the density of the solution calculated. From this pipette 2·0 c.c. of the solution were then accurately weighed into the dry, weighed cup of the Barcroft apparatus and about 0·3–0·4 c.c. accurately weighed into a weighed 50 c.c. graduated flask.

The glass standard = cd, where c = number of grams of oxyhaemoglobin in one litre of the

solution in the colorimeter cup as determined by the Barcroft apparatus, and d = colorimeter reading in cm.

The values of cd in five successive determinations were:

Fresh solution.	After thorough saturation with oxygen for 3–4 hours.
1·04	1·11
	1·10
1·06	1·12
1·07	1·11
1·05	1·11

A separate test showed the colorimeter to be self-consistent for known solutions of oxyhaemoglobin over a range of relative concentrations down to one-half of that of the strongest solution within one p.c. of the mean value.

For routine determinations 0·05 c.c. blood is added to 8·0 c.c. ammonia. For a modern instrument requiring less solution 0·02 c.c. blood and 2·5 c.c. ammonia would suffice.

It would thus appear:

1. The Barcroft apparatus is self-consistent when used on solutions of oxyhaemoglobin and apparent inconsistencies are due to incomplete oxygenation.

2. The simple and inexpensive system of colorimetry proposed is adequate for those purposes for which colorimetry could be considered sufficiently exact. This applies particularly to hospital laboratories and similar places where the work is performed by persons untrained in elaborate gas-analysis or the use of photo-electric methods.

3. Any gas-analysis method is apt to measure the oxygen content of the blood rather than its total oxygen capacity. Unless adequate precautions are adopted there is no reason why these should be exactly equal.

SUMMARY.

An inexpensive colorimetric method is outlined for the direct determination of oxyhaemoglobin using green light and a permanent glass standard.

REFERENCES.

Barcroft, J. and Roberts, F. (1909): J. Physiol., 39, p. 429.
Barcroft, J. *et al.* (1928): *Ibid.*, 66, p. 262.
Haldane, J. S. (1900): *Ibid.*, 25, p. 295.
Holden, H. F. (1943): Austral. J. exp. Biol., 21, p. 9.
Report on Haemoglobinometers to the British Medical Research Council (1943): Brit. med. J. (1943), p. 209.
Van Slyke, D. D. and Neill, J. M. (1924): J. biol. Chem., 61, p. 523.

A NEW SALMONELLA TYPE: SALMONELLA ADELAIDE

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Salmonella adelaide was isolated from a fatal case of enteritis in a man. It was found in the faeces during life and in the liver and spleen *post-mortem*. The morphological and cultural characteristics of the organisms place it in the salmonella group. It is a Gram-negative, motile bacillus growing readily on unenriched media, not forming indol, fermenting glucose, mannitol, dulcitol, maltose, arabinose, rhamnose, xylose, galactose and fructose with production of acid and gas, not fermenting lactose, sucrose, sorbitol, inositol, raffinose, salicin, starch, dextrin and glycogen, and is Stern glycerol-positive.

SEROLOGICAL WORK.

O Antigens.

Slide agglutination tests were negative with *S. adelaide* and O sera prepared against steamed suspensions of the following organisms:

S. paratyphi A I, II, XII	*S. aberdeen* XI	*S. kentucky* VIII, XX
S. abortus-ovis IV, XII	*S. havana* I, XIII, XXIII	*S. minnesota* XXI, XXVI
S. stanley IV, V, VII	*S. poona* XIII, XXII	*S. carrau* VI, XIV, XXIV
S. paratyphi C VI, VII	*S. kirkee* XVII	*S. schleissheim* IV, XXVII, XII
S. enteritidis I, IX, XII	*S. cerro*, XVIII	*S. tel-aviv* XXVIII
S. london III, X, XXVI	*S. senftenberg* I, III, XIX	*S. ballerup* XXIX

Weakly positive slide agglutination occurred with O sera prepared against the following organisms:

S. onderstepoort I, VI, XIV, XXV	*S. new-brunswick* III, XV
S. hvittingfoss XVI	*S. glostrup* VI, VIII

Tube agglutination tests with *S. adelaide* and the same set of O sera gave a weakly positive reaction only with *S. glostrup* serum. All the positive reactions on the slide or in the tube were too weak to indicate any major antigenic relationships.

An O serum prepared against a steamed suspension of *S. adelaide* did not agglutinate any of the salmonella homologous to the sera listed above. This confirms the previous result that the O antigen of *S. adelaide* is not related to any O antigen included in the test. Unfortunately no organisms or sera were available for O antigens XXX to XXXIV. Of these antigens XXXI and XXXII occur in coliform types and XXXIII in paracolon types (Peluffo, Edwards and Bruner, 1942) and on this account are unlikely to be found in *S. adelaide*. The remaining antigens occur in *S. urbana* XXX and *S. illinois* (III), (XV), XXXIV. *S. adelaide* contains neither III nor XV; if we consider how frequently the same set of antigenic components appear together, the presence of XXXIV in *S. adelaide* becomes unlikely in the absence of the accompanying factors. *S. adelaide* may contain XXX for which no tests were made.

No Vi antigen was detected by slide or tube agglutination with serum prepared against the V form of *S. ballerup*.

H. Antigens.

S. adelaide is monophasic; it contains the antigenic complex *g* which has never been found in a diphasic organism.

An H. suspension of *S. adelaide* (formolized broth culture) did not agglutinate with H. sera prepared against formolized broth cultures of the following organisms:

S. paratyphi A a	*S. abortus-equi* enx	*S. dar-es-salaam*, phase 1, lw
S. paratyphi B, phase 1, b	*S. typhi-murium*, phase 1, i	*S. oranienberg* mt
S. paratyphi C, phase 1, c	*S. thompson*, phase 1, k	*S. bareilly*, phase 1, y
S. typhi d	*S. london*, phase 1, lv	*S. poona*, phase 1, z
S. reading, phase 1, eh	*S. heidelberg*, phase 1, r	

Agglutination occurred with H sera prepared against the following organisms:

S. derby fg	*S. rostock* gpu	*S. montevideo* gms
S. enteritidis gm	*S. moscow* gq	*S. essen* gm
S. dublin gp	*S. senftenberg* gst	

H. suspensions of *S. cerro* z_4z_{23}, *S. ballerup* z_{14}, *S. kentucky*, phase 2, z_6, *S. uganda*, phase 1, lz_{13}, *S. glostrup* phase 1, z_{10}, *S. glostrup* phase 2, enz_{15} and the organisms homologous to the H sera listed above did not agglutinate with an H. serum prepared against *S. adelaide*. This serum agglutinated H suspensions of the following organisms:

S. derby fg	*S. moscow* gq	*S. havana* fg
S. enteritidis gm	*S. senftenberg* gst	*S. berta* fgt
S. rostock gpu	*S. essen* gm	
S. dublin gp	*S. budapest* gt	

It is evident that *S. adelaide* contains g and possibly f, m, p, q, s, t, u; but all tests with *S. oranienberg* mt were negative. To test for the remaining factors the following monospecific, absorbed sera were used:

f. *S. derby* serum fg absorbed twice with *S. essen* gm and *S. budapest* gt.
p. *S. dublin* serum gp absorbed twice with *S. enteritis* gm.
q. *S. moscow* serum gq absorbed twice with *S. enteritidis* gm.
s. *S. montevideo* serum gms absorbed twice with *S. enteritidis* gm.
pu. *S. rostock* serum gpu absorbed twice with *S. enteritidis* gm.

To prepare these sera, the absorbing organism was washed off agar with a suitable dilution of the serum (Kauffmann, 1939) and held at 50° C. for one hour (Edwards and Bruner, 1942) after which the mixture was centrifuged; more agar cultures were washed down with the supernatant which was again held at 50° C. for one hour and then centrifuged. The supernatant was the monospecific serum.

The H. suspension of *S. adelaide* agglutinated only with the f serum. The factor f is one of the rarer H components and occurs only in *S. derby* fg, *S. berga* fgt and *S. havana* fg. It is always associated with g which is recognized as a complex antigen, parts of which are missing in some organisms recorded as possessing g. To determine the relationship of the fg in *S. adelaide* to that in the other organisms, absorption tests were done. The results are recorded in Table 1.

TABLE 1.

Agglutination absorption tests.

	Sera.				
Suspensions.	Derby.	Adelaide absorbed with Derby and Enteritidis.	Adelaide absorbed with Havana.	Berta.	Derby absorbed with Adelaide.
Adelaide	6,400	800	400	3,200	0
Derby	0	0	0	0	0
Enteritidis	3,200	0	0	3,200	not done
Essen	3,200	0	0	3,200	0
Budapest	3,200	0	0	3,200	0
Rostock	0	0	0	0	not done
Moscow	800	0	0	3,200	not done
Montevideo	0	0	0	400	not done
Havana	6,400	0	0	3,200	not done
Berta	400	partial 400	400	0	not done

0 = no agglutination at 400.

The fg of *S. derby* and *S. berta* is not the same as the fg of *S. adelaide* as neither of these organisms appreciably reduced the titre of *S. adelaide* serum; but *S. adelaide* absorbed all the agglutinins from *S. derby* serum showing that the complete fg of *S. derby* is present in *S. adelaide* which contains an additional factor. A much closer relationship exists between the fg of *S. adelaide* and *S. havana* which removes most of the agglutinins from *S. adelaide* serum leaving only a low titre of residual agglutinins for the homologous organisms and *S. berta*. The same effect is produced by a double absorption with *S. derby* and *S. enteritidis*. Therefore, the fg of *S. adelaide* contains all the antigenic factors of *S. derby*, the g of which must be deficient in part of the complex. However, the g of *S. enteritidis* combined with the fg of *S. derby* is sufficient to remove all agglutinins for g or fg-containing organisms, but still does not exhaust the serum of agglutinins for *S. adelaide*. The antigen responsible for this residual reaction is also present to some extent in *S. berta*. The introduction of a new symbol for this antigen does not seem war-

ranted in view of the low titre of its antibodies and the complexity of g of which it may be another portion. It is therefore represented by a series of dots as used in other salmonella formulae, thus fg. . . .

DISCUSSION.

On account of its antigenic components, *S. adelaide* is a new salmonella type. Its O antigen is not related to any O antigen included in my tests. Unfortunately XXX was not available. The O antigen of *S. adelaide* may therefore be represented as a new factor or may prove to be XXX. The combination of either of these factors with the flagellar components f,g . . . constitutes a new antigenic formula. The antigen f has only been described three times before, namely in *S. derby* I, IV: f g . . ., *S. berta* IX: f g t . . . and *S. havana* I, XIII, XXIII: f g . . . By cross absorption tests, *S. adelaide* contains the whole antigenic complex of *S. derby* as well as another component, which appears to be a fraction of g present in *S. enteritidis* but lacking in *S. derby*. The *S. havana* f g is much more closely related to the *S. adelaide* f g and absorbs from *S. adelaide* serum all but a small residue of agglutinins for the homologous organism and *S. berta*. A double absorption with *S. derby* and *S. enteritidis* has the same effect. Therefore *S. adelaide* possesses a complex g comparable with that of *S. enteritidis* and *S. havana* as well as a minor H antigen (also present in *S. berta*) which may belong to the g complex or may be a separate entity and which is represented by the dots in the formula f g . . . *S. adelaide* has been sent to Dr. P. R. Edwards for further tests.

SUMMARY.

A new salmonella type, *S. adelaide*, is described and for it the antigenic formula XXXV: f g . . . is suggested.

REFERENCES.

Edwards, P. R. and Bruner, D. W. (1942): (Station) Circular 54, University of Kentucky, Agric. exp. Station.
Kauffmann, F. (1939): Actapath. et microbiol. Scandinav., 16, p. 278.
Peluffo, C. A., Edwards, P. R. and Bruner, D. W. (1942): J. infect. Dis., 70, p. 185.

HYPERICIN AND A NON-FLUORESCENT PHOTOSENSITIVE PIGMENT FROM ST. JOHN'S WORT *(HYPERICUM PERFORATUM)*

by ROBERT CECIL BETTY AND VICTOR MARTIN TRIKOJUS.[1]

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INTRODUCTION.

It has long been known that the St. John's Wort (*Hypericum perforatum*) causes photosensitization when ingested by animals with unpigmented skins; this has frequently led, in the less temperate climates, to serious losses of stock (see Blum, 1941). The leaves, stalks and flowers were found to yield a highly-fluorescent red pigment (or mixture of pigments) which brought about an analogous reaction when injected into animals (Ioannidès, 1930; Horsley, 1934; Brockmann *et al.*, 1939).

The exact structure of the pigment is as yet unknown; Cerny (1911) claimed to have identified the pigment, which he named *hypericin*, as chemically uniform, $C_{16}H_{10}O_5$, and he suggested that it belonged to the class of flavones. Ioannidès stated that he obtained the substance in the form of microscopic crystals, a result also claimed by Brockmann *et al.*, who, in the brief report of their work, mentioned the preparation of crystalline acetyl and benzoyl derivatives. These latter workers deduced the formula $C_{28}H_{16}O_8$ for hypericin, and suggested a hexahydroxy meso-dianthrone structure. However, Pace and MacKinney (1939, 1941) have recently brought forward evidence of the non-homogeneity of the fluorescent pigment fraction of *Hypericum perforatum*; two main products, each non-crystalline but chromatographically homogenous, designated as "hypericin Y" and "hypericin X", were isolated, the former analysing as $C_{29}H_{22}O_8$ and the latter less certainly as $C_{31}H_{28}O_9$. Unfortunately the biological properties of the two pigments were not reported by these workers.

The purpose of the present investigation (initiated in 1939) was to attempt the preparation of a more refined product than that obtained by Horsley in this Department, and to study its biological effects. A highly active fluorescent pigment was obtained by chromatographic adsorption methods and precipitation from phosphate buffer solution, but the material was not obtained definitely crystalline.[2] During the course of these experiments it was observed that, when all procedures were carried out in the dark or in subdued artificial light, the solution of the crude-pigment fraction, in contrast to that from the control extractions carried out in daylight, was purplish in colour and of much reduced fluorescence. This was found to result from the presence of an appreciable quantity of a photosensitive bluish-purple non-fluorescent pigment, which could be readily separated from the fluorescent material by selective adsorption, but into which it appeared to be com-

[1] One of us (R.C.B.) is indebted to the Commonwealth Research Grant Committee (University of Sydney) for a personal grant during 1940.

[2] For clarity this pigment is referred to in the text as hypericin (L) whilst that separated from the precursor during extraction in the dark is designated hypericin (D). If the latter and the irradiated precursor prove to be different pigments, then hypericin (L) would, from our results, be most likely a mixture of the two. Hypericin (D) would possibly also be contaminated, in this event, by irradiated precursor, since some change during the extraction procedure would be difficult to prevent.

pletely transformed after short irradiation in daylight (Betty and Trikojus, 1941). Young plant material collected in the Spring contained a greater proportion of the non-fluorescent "precursor" than fluorescent pigment, whilst material collected in the following Autumn from the same area contained a negligible amount of the precursor.

It has not been possible to compare chemically the irradiated precursor and the purified fluorescent material accompanying it (hypericin (D)[2]), owing to insufficient material. The absorption spectra in the visual region of these two red-fluorescent pigments, are, however, very similar, whilst a mixture of the two could not be differentiated by adsorption on the calcium carbonate used in our experiments, or on aluminium oxide. On the other hand, when injected into rats, even in doses of comparable intensity of fluorescence, the irradiated precursor exhibited an appreciably lower potency than the fluorescent pigment, together with some differentiation in its action. These observations are not inconsistent with the conclusion of Pace and MacKinney as to the existence of a group of closely related compounds in the red fluorescent pigment fraction of extracts of St. John's Wort.

The natural occurrence of a precursor of this group provides further evidence of the close structural similarity between these pigments and those of the mould *Penicilliopsis clavariaeformis* (Oxford and Raistrick, 1940). A comparison of the absorption spectra of our precursor and oxypenicilliopsin has been made. The curves obtained show a strong resemblance.

The actual structure of the hypericin group is still a matter for conjecture. Pace and MacKinney (1939, 1941; cf. also Oxford and Raistrick, 1940) favour a polyhydroxyhelianthrone structure (for "hypericin Y"), and this suggestion receives support from our further observation that hypericin breaks down progressively to non-fluorescent material on prolonged exposure to sunlight. A. G. Perkin and collaborators (e.g. 1924, 1929) have noted, under similar conditions, the conversion of simple hydroxyhelianthrones to naphthadianthrones.

PREPARATION OF PIGMENTS.

Hypericin (L).

The plant material used consisted mostly of young shoots collected September–October, 1939, in the Mudgee district (New South Wales). The green plant was air-dried in subdued light and mechanically disintegrated. This material (6·25 kg.) was covered with methanol (26 litres) and refluxed for 1·5 hrs. The extraction was repeated three times with a further 18 litres of the solvent. The combined extracts were filtered, reduced in volume to one-fourth, made up to the original volume with water and allowed to stand. After filtration, the turbid filtrate was shaken three times with benzene (500 ml. per 16 litres filtrate), some powdered salt being added to inhibit emulsification. A clear reddish brown fluorescent solution free from chlorophyll was thus obtained. The solution was then shaken with excess powdered salt and extracted four times with ethyl acetate (1,000, 500, 500, 500 mls. per 16 litres solution). The combined ethyl acetate extracts (4·5 litres) of a reddish brown colour and strong red fluorescence, were washed with successive small portions of a saturated aqueous sodium carbonate, until the washings were almost colourless, and finally with water. The extract, which had now lost its brownish tinge, and was a rich red colour, was dried over anhydrous sodium sulphate and concentrated in an atmosphere of nitrogen to about one-tenth volume. The concentrate (350 ml.) was mixed with aqueous disodium hydrogen phosphate solution (0·3 p.c.; 1 litre) and ether (1 litre), thoroughly shaken and allowed to separate. The dark reddish purple lower layer was removed and the ethyl acetate: ether layer shaken with more phosphate, and a further volume of ether. This process was repeated until the ethyl acetate: ether layer was yellow in colour. The combined phosphate solution was then filtered and the pigment precipitated by adjusting the pH to about 2 with dilute sulphuric acid. The pigment was then centrifuged, washed with a little water and dried *in vacuo*. Further purification was effected by the use of chromatography. After many trials with several adsorption media, including alumina (Merck "nach Brockmann"), calcium carbonate (A.R., "B.D.H." or "H. and W.") was finally chosen. The pigment was adsorbed on the column from 2: 1 ethyl acetate: benzene, developed with a 1: 2 proportion of these solvents, and the central part of the reddish band eluted with ethanol. This process was repeated several times, the pigment finally being precipitated as a black powder, from a concentrated solution in ethanol, by the addition of petroleum ether.

The hypericin dissolved in alkali to a green solution but it was soluble sparingly in most organic solvents. A dilute solution could be obtained, however, in ethyl acetate or ethanol (and methanol), glacial acetic acid and pyridine with characteristic red fluorescence. Its solution in phosphate was red but non-fluorescent. The pigment appeared to be chromatographically homogeneous, but it could not be obtained crystalline with certainty. Moreover, on analysis a residual alkaline ash was consistently obtained with different specimens. The absorption spectrum in the visual region of a solution in ethyl acetate showed maxima at 593 and 551 mμ.

The hypericin obtained as outlined above was mostly used in chemical tests and in an effort to attain greater purity. Although it readily reacted with acetic anhydride and benzoyl chloride to give yellow or brown derivatives no single crystalline product could be isolated. The hypericin used in the animal experiments was that separated from the precursor and further purified (see below).

Decomposition of hypericin in sunlight. Hypericin (10 mg.) prepared as above and purified by a further precipitation from buffer solution was taken up as completely as possible (9 mg.) by successive extraction with a total of 90 ml. ethanol plus a few drops of pyridine. 40 ml. of this solution were kept in the dark to serve as control whilst the residue was exposed to external sunlight for a period of 82 days in a sealed clear glass bottle. At intervals, as set out in Fig. 1, the fluorescence of the irradiated solution was compared with that of the control by means of the Fluoroquant (Minibeck, 1937) and its relative hypericin content calculated. The exposed hypericin solution gradually assumed a brown colour in transmitted light, until at the conclusion of the experiment the brown solution contained a fine dark precipitate (which gave a green colour with concentrated sulphuric acid), and was practically non-fluorescent in ultra-violet light.

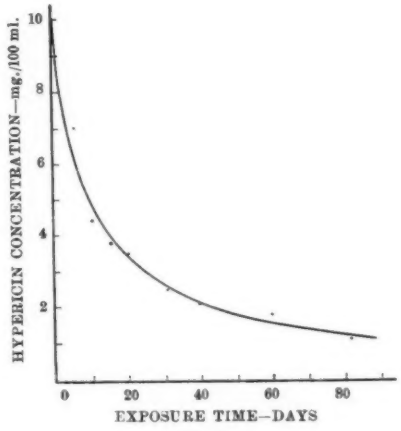

Fig. 1.

The *precursor and hypericin (D).* If at that stage in the isolation of hypericin (L) described above, where the ethyl acetate was washed with saturated sodium carbonate solution, all prior operations had been conducted in the dark or in subdued orange or red light, the extract exhibited only a mild red fluorescence and was a purple colour in transmitted light. Further purification and separation of the two main pigment components, hypericin (D) and the precursor, were effected by diluting the dried and concentrated ethyl acetate extract with benzene (respectively 3: 1) and adsorbing onto calcium carbonate. The precursor formed a wide purplish zone below the red band of the fluorescent pigment. Each zone was eluted with ethanol and separately adsorbed several times onto carbonate from ethyl acetate: benzene (3: 1 or 2: 1). Finally, the hypericin fraction was precipitated from ethyl acetate with petroleum ether, then extracted twice from its solution in ethyl acetate: ether with 0·3 p.c. aqueous disodium hydrogen phosphate and precipitated by adjusting the deep red phosphate solution to pH 2 with 2N sulphuric acid. The black precipitate was centrifuged, washed twice with water and dried in a vacuum desiccator. The low yield (75 mg. from 4·2 kg. of young plant material, plus 6 kg. of the Autumn-collected material containing very little precursor) was largely due to the narrow cut made in the appropriate zone during the successive adsorptions. This material represented the hypericin used in the animal experiments.

The ethyl acetate solution of the uniform zone in the final precursor column was precipitated with petroleum ether—as a purple solid—and dried *in vacuo.* Yield 1·18 gm. (containing inorganic impurity). Phosphate insoluble pigments and other impurities were removed by dissolving the precursor in 0·3 p.c. aqueous disodium hydrogen phosphate from ethyl acetate: ether solution and precipitating from the purple red phosphate solution with 2N sulphuric acid. After washing with water and drying *in vacuo* the precipitate weighed 146 mg. This material was combined with a residual 45 mg. of material at this stage from previous batches and exhaustively extracted in a micro-Soxhlet apparatus with peroxide-free ether. The ether extract was evaporated and the residue dissolved in 2: 1 ethyl acetate: benzene and adsorbed on calcium carbonate. The top portion of the uniform band was discarded and the ethanol eluate of the residue taken up in ethyl acetate, precipitated with petroleum ether, centrifuged and dried (77 mg.). The pigment was now only very sparingly soluble in ether. A portion was taken up as far as possible in ether, filtered, the ether removed and the residue dissolved in ethyl acetate and precipitated with six volumes of petroleum ether. An inorganic impurity however, still persisted (Micro analysis: C. = 61·0; 61·4; H = 5·56; 5·42; Ash = 1·58, 1·50 p.c.). The remainder was dissolved

as far as possible in boiling freshly distilled nitrobenzene (20 ml.), centrifuged and the pigment precipitated by adding petroleum ether (100 ml.). After centrifuging, washing with petroleum ether several times and drying *in vacuo*, 46 mg. were collected. This material provided the precursor and irradiated precursor for the animal experiments.

The precursor formed a non-crystalline reddish purple powder, more soluble than hypericin in alcohols, ethyl acetate or phosphate buffer. It dissolved in ethanol to a non-fluorescent bluish red or bluish purple solution which rapidly developed a fiery red fluorescence on exposure to sunlight. This change, as with the conversion of oxypenicilliopsin into irradiated oxypenicilliopsin (Oxford and Raistrick, 1940), does not involve oxidation. It occurred at the same rate in two ethyl acetate solutions each containing 0·5 mg. of the pigment in 10 ml., through which air and nitrogen were bubbled respectively, and was complete after one hour.

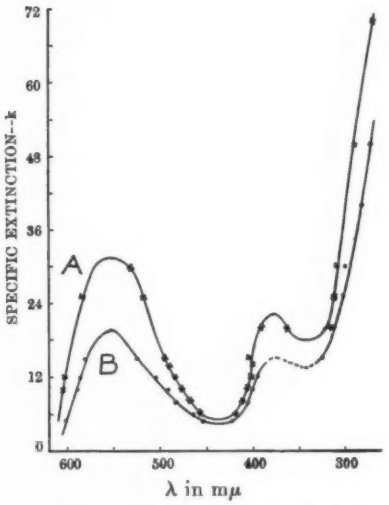

Fig. 2. Absorption curves of oxypenicilliopsin (A), and the precursor (B) in ethanolic solution.

As found subsequently the precursor absorbs only weakly in the red part of the spectrum. Bluish-green light, as in the following experiment, effected the conversion much more rapidly than red.

Two tubes each containing 10 ml. of a 1/50,000 solution of the precursor in ethyl acetate were placed at a distance of 15 cm. from the centre of a 100 watt lamp screened by cells containing dilute nickel sulphate solution. Two filters were interposed respectively between the lamp and the two tubes, one a red filter, Schott and Gen *RG1*, and the other bluish-green, Schott and Gen *BG18*. The set-up was enclosed in a blackened box in a dark-room. The solutions were compared at intervals with a non-illuminated control. The light passing through the blue filter was found to have effected the change completely in 30 minutes, whilst in the other case 5½ hours exposure was necessary.

Comparison of the Absorption Curves of the Precursor and Oxypenicilliopsin.[3]

Dr. J. E. Mills of the Department of Chemistry kindly made a preliminary study of the absorption curves of ethanolic solutions of oxypenicilliopsin and the precursor (Fig. 2) by means of the quartz spectrograph in conjunction with the Hilger rotating sector photometer and a condensed tungsten steel spark as light source. The specific extinction $k = \frac{\log I_0/I}{cd}$ was plotted against the wave length in millimicrons, where I_0 = incident intensity; I = transmitted intensity; c = concentration in grams per litre; d = thickness in centimetres.

The general forms of the curves run closely parallel, although the precursor shows appreciably lower absorptive power at equivalent dilution.

The irradiated precursor. The red-fluorescent pigment obtained by irradiation of the precursor appeared to be indistinguishable spectroscopically from hypericin (D), although a detailed comparison was not made. Moreover, the two fluorescent pigments either separately or mixed gave a single zone when adsorbed from ethyl acetate: benzene solution onto a column of calcium carbonate or aluminium oxide (Merck "nach Brockmann"). In the visible region it showed the same characteristic adsorption bands with maxima at 593 and 551 mμ in ethyl acetate solution. However, a solution of a given weight of the precursor gave, after exposure to daylight, a similar but less intense fluorescence than that given by a solution of the same weight of the hypericin specimen. In ethanol the weight ratio for equivalent fluorescence was approximately 2·1: 1.

When tested on rats, as detailed later in the paper, both pigments produced qualitatively similar reactions. However, more severe symptoms (involving the death of the animals) resulted from the injection of 1 mg. (or even 0·5 mg.) of the hypericin specimen, than from 3 mg. of the irradiated precursor. The work of Pace and MacKinney, and Oxford and Raistrick has indicated more exactly the natural occurrence of a group of very closely related pigments of this class, and it is conceivable that the members of this group would show a gradation or differentiation in their physiological effects.

[3] We are indebted to Dr. A. E. Oxford (London) for the specimen of oxypenicilliopsin.

ANIMAL EXPERIMENTS.

Young Wistar rats of both sexes were housed separately in wide-meshed wire cages and maintained on a balanced dry diet plus water. Weighed quantities of the pigments were dissolved as far as possible in 1–2 ml. M/15 aqueous disodium hydrogen phosphate, and the suspension thus obtained injected subcutaneously in the dorsal region unless otherwise stated, and the animals, together with the controls receiving phosphate solution only, were immediately and equally exposed to direct sunlight and their reactions noted. During the exposure periods only water was available to the animals. Subsequent to the irradiation treatment (the period of which varied in the several experimental groups) the surviving animals were observed away from direct sunlight up to the time of complete recovery. Following on a preliminary test (Experiment No. H) to ascertain the potency of the hypericin sample used, six experiments were carried out to study the comparative effects of hypericin D ("Hy."), the precursor ("Pr.") and the irradiated precursor ("Ir. Pr.") with the following general results, particular data being set out in Table 1. The observations made during Experiment A are presented in greatest detail.

Experiment A. After 1–2 hours exposure on the first day, rats A7–10 (hypericin injected) showed signs of irritation by scratching and rubbing at the head and sides of the body, and by excitedly jumping at irregular intervals. The precursor-treated rats and the controls showed no unusual symptoms.

TABLE 1.

Summary of experiments on the effect of injection of hypericin D (Hy.), precursor (Pr.) and irradiated precursor (Ir. Pr.).

Expt. No. and date.	Rat No.	Weight (g.).	Injection.*	Exposure to sunlight. Days.	Hours.	Whether fatal.
H (from 16-5-41)	H1	194	1·0 mg. Hy.	8	37	+ (4 days)
	H2	168	" "			+ (11 days)
	H3	202	Phosphate			—
	H4	208	controls			—
A (from 27-5-41)	A1	135	Phosphate	5	20	—
	A2	150	controls			—
	A3	146	2·0 mg. Pr.			—
	A4	163	" "			—
	A5	138	1·0 mg. Pr.			—
	A6	121	" "			—
	A7	146	2·0 mg. Hy.			+ (2 days)
	A8	165	" "			+ (2 days)
	A9	136	1·0 mg. Hy.			+ (2 days)
	A10	119	" "			+ (2 days)
C (from 18-9-41)	C1	167	Phosphate	9	53	—
	C2	184	controls			—
	C3	150	1·0 mg. Pr.			—
	C4	156				—
	C5	133				—
	C6	150	1·0 mg. Hy.			+ (2 days)
	C7	132				+ (2 days)
	C8	155				+ (14 days)
	C9	192	Phosphate	Not exposed to sunlight		—
	C10	178	controls			—
	C11	150	1·0 mg. Pr.			—
	C12	119				—
	C13	162				—
	C14	136	1·0 mg. Hy.			—
	C15	154				—
	C16	129				—

TABLE 1 (continued)

Expt. No. and date.	Rat No.	Weight (g.).	Injection.*	Exposure to sunlight. Days.	Hours.	Whether fatal.
	D1	216	Phosphate			—
	D2	218	controls			—
	D3	160	0·5 mg. Pr.			—
	D4	173	" "			—
	D5	163	0·5 mg. Pr.			—
	D6	115	(abdomen)			—
D	D7	135	0·5 mg. Hy.			+ (16 days)
(from 30-9-41)	D8	129	" "	6	24	+ (11 days)
	D9	131	0·5 mg. Hy.			+ (by accident)
	D10	161	(abdomen)			—
	D11	140	1·0 mg. Ir. Pr.			—
	D12	151	" " "			—
	D13	125	0·5 mg. Ir. Pr.			—
	D14	148	" " "			—
	E1	253	Phosphate			—
	E2	233	controls			—
E	E3	164	1·0 mg. Hy.			+ (16 days)
(from 27-10-41)	E4	207	" "	8	48	+ (3 days)
	E5	205	2·1 mg. Ir. Pr.			—
	E6	186	" " "			—
	F1	244	E2			—
	F2	260	E1			—
	F3	184	1·0 mg. Hy.			+ (4 days)
F	F4	120	" "			+ (3 days)
(from 10-11-41)	F5	174	3·0 mg. Ir. Pr.	14	88	—
	F6	158	" " "			—
	F7	168	3·0 mg. Pr.			—
	F8	166	" "			—
B	B1	120	Phosphate			—
(from 14-7-41)			control	7	20	
	B2	130	1·0 mg. Hy.	(Artificial	light)	—

* Subcutaneously (in dorsal region unless otherwise stated).

On the second day rats A7–10 became very excited immediately after exposure. A7 appeared to be the worst affected and would occasionally jump about in a frenzy, biting at the site of injection and shaking itself vigorously. After about 1½ hours a well-marked erythema was present on the ears of the four animals. Later in the day A7 developed an opaque film in both eyes. This was also noted in A9 (left eye) and A10 (both eyes). Group A3–6 (precursor-injected) in contrast to the control rats A1–2, also became restless soon after exposure, scratching at the back and head. A3 squealed and rushed about the cage biting its back. A slight erythematous condition also developed on the ears of this group, but the eyes were unaffected.

Rats A7, A9 and A10 died during the night of the 2nd day whilst A8 died on the morning of the third day before being exposed. At autopsy all animals showed marked erythema of the feet, ears and tail. The forefeet were dry and shrivelled whilst the skin of the ears was also in a dry condition. With the exception of A9 there was a marked oedema present in the region of the head and forelegs. The stomachs of all animals were empty although they had had access to food. The inner surface of the skin both in the dorsal and anterior parts of the body appeared bluish red in colour in contrast to the pale colour of the skin of a normal control. Some unabsorbed pigment was found near the site of injection. In contrast to the observations of Horsley (1934) blood was not noticed in the faeces of these animals, nor of those in any of the several experimental groups.

Irradiation of the precursor-treated rats was continued during the third and fourth days of the experiment and for a brief period on the sixth day. The animals continued to show marked irritation in the presence of sunlight and the erythematous condition became worse. The animals endeavoured to protect their hairless parts by shading the tail with the body and crouching with the head downwards away from the sun. (In general all the pigment-injected animals in this and the other experiments, apart from irregular frenzied jumps, adapted themselves to such a position

when in direct sunlight). After 6–8 days a hairless patch with a hard dry skin developed on each of the four animals near the site of injection, extending in the case of A3 from the middle of the back down to the left foreleg. From the 11th–17th days these dry areas became raw and moist. Subsequently the lesions formed hard scabs which sloughed off and after 24–35 days the affected areas were completely healed in all animals. From the 6th to 9th days the ear-tips of the four animals became shrivelled and gangrenous and later dropped off (with the exception of those of A5).

During the whole course of this experiment the two control animals showed no unusual symptoms other than a mild reddening of the ears which rapidly cleared, when removed from direct sunlight.

Experiments C, D, E. F. In view of the rapid death of the four hypericin-treated rats in Experiment A, the experiment was repeated with a control group maintained in a darkened room (Experiment C). These animals C9–16 showed no unusual symptoms whatever throughout the experiment, whereas the pigment-injected animals in the irradiated group reacted similarly to those in Experiment A, although one of the hypericin-injected rats, which was blind from the 7th day, survived for 14 days. Both the precursor- and hypericin-injected rats showed reactions on the first day of exposure to sunlight (within 20 minutes and 5 minutes respectively).

In Experiments D, E, and F the precursor, both before and after irradiation *in vitro*, was compared with hypericin. As mentioned previously 2·1 mg. of the precursor exhibited after irradiation an intensity of fluorescence approximately equivalent to that of 1·0 mg. of hypericin, and this quantity was used, after irradiation in ethyl acetate and removal of the solvent *in vacuo*, in Experiment E, whilst 3·0 mg. were injected in Experiment F (see Table 1). The observations made in these three experiments are considered, together with those of Experiments A and C, in the following comparative summary.

General observations. In general, all the pigment-injected rats showed signs of irritation on the first day of exposure to sunlight at periods varying from 5 minutes to 2 hours with the exception of those treated with the precursor in Experiments A and D, which reacted on the second day. The hypericin-treated rats were usually first affected.

Of eleven rats injected in the dorsal region with 1 mg., and two with 2 mg. hypericin, eight died after two days, two after four days whilst the remaining three survived for 11–16 days, as did two rats injected with 0·5 mg. By the third day of exposure, if the hypericin-treated rat still lived, a marked unilateral or bilateral oedematous swelling developed in the region of the head, shoulders and forelegs. In those cases where the animals survived for 11–16 days the ears became dark red and shrivelled and later bluish and gangrenous. After 7–8 days the eyes became opaque and the eyelids matted, resulting in blindness of the animals. Furthermore, a small hairless patch developed on the neck near the site of injection, but this was never as extensive nor as ulcerated as with the precursor-injected rats at the same period. At death the animals were in a very poor condition. Two rats received 0·5 mg. hypericin injected subcutaneously in the abdominal region, the site of injection being thus protected from direct sunlight. One of these animals (D9) died accidentally on the third day, the other (D10) survived the conclusion of the experiment at 30 days. This animal showed qualitatively the same responses as the others injected with hypericin except that a hairless dry patch developed near the site of injection (from the 7th day) as well as on the back (from 14th day). Both these areas were normal by the 30th day.

On irradiating those animals injected with 1·0 mg. precursor the early reactions were qualitatively similar to those in the hypericin-treated rats. Later a hairless patch developed on the back (5–8 days) which rapidly extended, in some animals down the side of the foreleg. These areas became raw, with a serous exudation (11–17 days), but subsequently dried and returned, without treatment, to a normal condition. After 6–8 days the ear-tips were hard and shrivelled, and dropped off in most cases after 16–24 days. None of the precursor-injected rats died, even those given 3·0 mg. The latter developed swollen heads and forelegs (3–4 days) but otherwise behaved like those injected with 1·0 mg. Rats injected with 0·5 mg. precursor showed only a mild reaction to sunlight and developed only small dry hairless patches whilst those receiving 0·5 mg. in the abdominal area showed practically no symptoms (Experiment D).

With the irradiated precursor the symptoms were similar to those produced by the same weight of the non-irradiated pigment. None of the animals died, although as much as 2·1 mg. and 3 mg. of the precursor were irradiated to full fluorescence and then injected (Experiments E and F).

The surviving rats in Experiment F were sacrificed after 17 days (one day more than the maximum survival period of the hypericin injected rats in all experiments) and it was noted that a proportion of the pigments (precursor and exposed precursor) remained unabsorbed at the site of injection (confirmed by extraction of the surrounding tissue).

Effect of artificial light (Experiment B). Two rats, one receiving an injection of hypericin (1 mg.) and the other serving as a control, were exposed to the light from two 500-watt photo-flood lamps and one ordinary 500-watt lamp, at a distance of about one metre. The control showed no unusual symptoms, whilst the pigment-injected animal exhibited only a mild reaction compared with the animals exposed to sunlight.

SUMMARY.

By carrying out all processes in the dark or in subdued artificial light, *Hypericum perforatum* has been shown to yield a non-fluorescent pigment (which rapidly becomes fluorescent when solutions of it are exposed to daylight) and a fluorescent pigment (hypericin (D)), which were easily separated by adsorption methods. By extraction in daylight, a red-fluorescent pigment (hypericin (L)) only was obtained.

When tested on rats, the red-fluorescent pigment (hypericin (D)) proved to be more potent, and showed some differentiation in its action, compared with the non-fluorescent pigment, both before and after its transformation by light.

Acknowledgment. The authors are indebted to Professor C. G. Lambie for his continued interest in this investigation.

REFERENCES.

Betty, R. C. and Trikojus, V. M. (1941): Austral. J. Sci., 3, p. 100.
Blum, F. B. (1941): Photodynamic Action and Diseases Caused by Light, New York.
Brockmann, H., Haschad, M. N., Maier, K. and Pohl, F. (1939): Naturwissenschaften, 27, p. 550.
Cerny, C. (1911): Z. physiol. Chem., 78, p. 371.
Haller, J. W. and Perkin, A. G. (1924): J. chem. Soc., 125, p. 231.
Hardacre, R. W. and Perkin, A. G. (1929): *Ibid.*, p. 180.
Horsley, C. H. (1934): J. Pharm. exp. Ther., 50, p. 310.
Ioannidès, Z. M. (1930): Compt. rend. Soc. biol., 105, p. 349.
Minibeck, H. (1937): Biochem. Z., 293, p. 219.
Oxford, A. E. and Raistrick, H. (1940): Biochem. J., 34, p. 790
Pace, N. and MacKinney, G. (1939): J. Amer. chem. Soc., 61, p 3594.
Pace, N. and MacKinney, G. (1941): *Ibid.*, 63, p. 2570.

THE PHARMACOLOGY AND TOXICITY OF ALSTONIA ALKALOIDS

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Alstonia is a genus widely distributed in Northern Australia. The bark of *A. constricta* and *A. scholaris* has been advocated at various times by Bancroft and others as an alternative to cinchona in malaria and other febrile conditions.

Interest in the subject has recently been revived in Australia because preliminary work in this laboratory had shown that the contained alkaloids possessed many properties in common with quinine and so might serve as antimalarials.

Chemical investigation of the alkaloidal constituents has been undertaken by Sharp (1938) and Elderfield (1942). The bark contains a mixture of alkaloids which are rather ill defined and unstable. Mixed sulphates of *A. constricta* var. mollis (prepared by Mr. Bennett of the Developmental Section of Elliots & Australian Drugs Pty. Ltd. and supplied to us through the courtesy of Professor V. M. Trikojus) were used in the present experiments and are referred to throughout this paper as alstonia alkaloids.

Our aim was to investigate the pharmacology and toxicity preparatory to clinical trials which were to be carried out under the direction of Professor Harvey Sutton.

In all cases it was thought desirable to compare the properties of alstonia alkaloids with those of quinine, especially the toxicity, as previous experience had led us to believe that Australian animals usually gave an L.D. 50 for other drugs two to three times higher than overseas values.

The only previous pharmacological work had shown that alstonia alkaloids possessed the power of causing contraction of the uterus (Sharp, 1934).

EXPERIMENTAL.

The Isolated Intestine of the Rat, Rabbit, and Guinea-pig. (Magnus Method).

Volume of bath, 20 ml. Firstly the effect of alstonia alkaloids on the smooth muscle was observed and then the alteration in response of the intestine to acetyl choline, adrenaline, barium and potassium, when it had previously been treated with alstonia alkaloids for a period of from 3–5 minutes.

Fig. 1. Rat. Isolated Intestine. Al. Alstonia sulphates 1·6 mg.

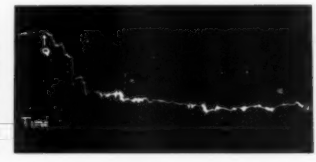

Fig. 2. Rat. Isolated Intestine. Q. Quinine bisulphate 0·6 mg. Time, 30 sec.

[1] This work has been aided by technical assistance provided by the National Health and Medical Research Council.

The following results were obtained, mainly with the rat's intestine which proved to be the most suitable.

Addition of 200 μg of the mixed sulphates of alstonia alkaloids to the bath caused a fall in tonus, a cessation of movement for a time and then, usually, with the tone still depressed, a rhythmical movement with increasing amplitude. Often the addition of alstonia alkaloids to a quiescent intestine would cause rhythmical movement. In all cases the results were similar to those obtained with quinine (Figs. 1 and 2).

Both quinine and alstonia alkaloids abolished the effect of acetyl choline and barium and lessened those of adrenaline and potassium. The inhibitory effect was not prolonged and disappeared a few minutes after the alkaloid had been washed out.

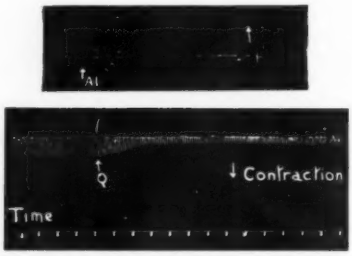

Fig. 3. Frog. Heart. Straub Cannula. Al. Alstonia sulphates 1: 5,000. Q. Quinine bisulphate 1: 10,000. Time, 30 sec.

Uterus.

(a) *Isolated uterus.* On the rat or guinea-pig uterus quinine and alstonia alkaloids sometimes cause a contraction and sometimes a relaxation, but on the same piece of uterus quinine and alstonia always gave a response of the same type.

(b) *Uterus* in situ. 20 mg. of quinine or alstonia alkaloids injected intravenously into a cat brought about marked contraction of the non-pregnant uterus. Whilst the toxicity tests were in progress it was noted that after the administration of alstonia two pregnant cats and a rabbit gave premature birth to their young.

Frog's Heart (Straub Preparation).

Quinine and alstonia alkaloids in a concentration of 1: 5,000 to 1: 10,000 brought about a decrease in amplitude without alteration in rate (Fig. 3).

Heart and Circulation in Intact Animal.

Cats were anaesthetized with nembutal and a little ether. Drugs were administered intravenously.

Alstonia alkaloids 3–5 mg./kg. bring about a sharp fall in blood pressure but very little alteration in heart rate. Larger doses are definitely toxic causing irregularities in rhythm, AV block, and sometimes ventricular fibrillation. The respiratory centre is very readily attacked, even by small doses and artificial respiration may have to be resorted to.

Quinine is much less active, 13–18 mg. being required to produce a fall in B.P. Also the toxic reactions are less marked.

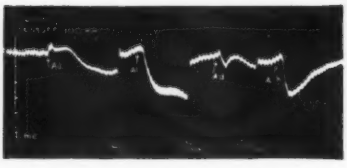

Fig. 4. Cat. Nembutal and Ether. Ad. Adrenaline 0·005 mg. Al. Alstonia sulphates 5 mg./kg. A.ch. Acetyl choline 0·001 mg. Time, 30 sec.

Fig. 5. Cat. Nembutal and Ether. Ad. Adrenaline 0·005 mg. A.ch. Acetyl choline 0·0001 mg. Q. Quinine bisulphate 17 mg/kg. Time, 30 sec.

About 5–10 minutes after the administration of alstonia alkaloids or quinine the injection of adrenaline brings about a fall in B.P. The conditions necessary to show this vaso-motor reversal are the same as those for yohimbine (Hutchinson, Shaw, Wragge, 1942). On several occasions cardiac irregularities were noted when adrenaline was injected about 10 minutes after the quinine. These did not occur if the adrenaline was administered simultaneously with the quinine.

The response to acetyl choline is usually lessened by the previous administration of alstonia alkaloids and quinine (Figs. 4 and 5).

Striated Muscle.

The technique is similar to that described by Brown (1938). Dogs were anaesthetized with Dial and ether. The contraction was measured by an isometric lever attached to the tendon of tibialis anticus. A cannula was inserted in a superficial branch of the popliteal artery, facing towards the heart. Before an injection was made the popliteal artery was clamped, the injection was then made and the clamp released. In this manner arterial injections could be made and the natural circulation maintained. The peroneal nerve was stimulated every 10 seconds by means of an induction coil.

Alstonia alkaloids 0·8 mg./kg. produced initially a fall in the tension developed followed by a very slight increase, less than 10 p.c. (Fig. 6).

After eserinization of the muscle the large increase in tension developed was reduced to about 20 p.c. of its original value (Fig. 7).

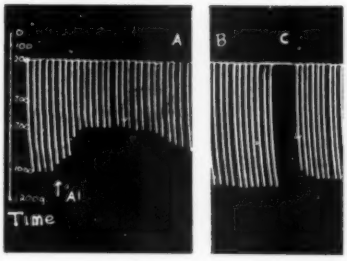

Fig. 6. Dog. Tibialis anticus. Al. Alstonia sulphates 0·8 mg./kg. Between A and B 2 min. C interval of 30 min. Time, 30 sec. Tension in grams.

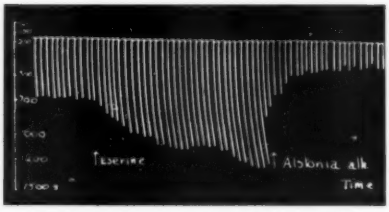

Fig. 7. Dog. Tibialis anticus. Eserine. 0·03 mg./kg. Eserine. Alstonia sulphates 0·3 mg./kg. Time, 30 sec. Tension in grams.

Both these results are similar to those produced by quinine (Harvey, 1939) except that quinine did not produce an initial lowering of tension developed and the increase usually amounted to about 30 p.c.

Toxicity Tests.

The results of these tests have been collected in Table 1. There are two series of experiments: (a) the administration of a single dose to determine the LD 50, (b) the administration of the drug over a period to determine the presence or absence of cumulative effects.

The experiments have brought to light several interesting points. By all methods of administration the toxicity of alstonia alkaloids and quinine shows a remarkable parallelism. Subcutaneous or intramuscular injection of alstonia alkaloids gives marked tissue damage, in this respect alstonia alkaloids are about 3–4 times as damaging as quinine. Intravenously alstonia is much more toxic than quinine.

REMARKS.

Very little has been published on the action of quinine on isolated organs. The above findings agree in the main with those of earlier workers (Sollman, 1936). However the vaso-motor reversal with quinine and the action on the cat's uterus *in vivo* have not been so definitely established before as in our experiments.

The results suggest that pharmacologically the alkaloids of *Alstonia* resemble those of cinchona and although the results of animal experiments indicate that it has little antimalarial activity, the resemblance is such as to suggest a close similarity of chemical structure and that further investigation along this line should be undertaken.

SUMMARY.

The action of the mixed alkaloids of *A. constricta* has been investigated on isolated intestine, uterus, heart, circulation and striated muscle. In all cases the actions resembled those of quinine.

A table of the toxicity to various animals is included.

TABLE 1.

Animal.	Administration.	Drug. Quinine.	Number of animals.	Alstonia.	Number of animals.	Number of daily doses for each drug.
*Acute**						
Mouse	Peritoneal	800	28	800	40	
	Hypodermic	1,000	15	1,000	15	
Rat	Peritoneal	300	9	500	20	
	Intramuscular	400	6	400	4	
	Hypodermic	—	—	200	18	
	Oral	500	4	850	8	
Guinea-pig	Intramuscular	500	12	500	16	
	Peritoneal	1,700	4	1,700	3	
	Oral	1,500	12	1,500	12	
Cat	Intravenous	25	8	15	8	
	Intramuscular	—	—	200	2	
Rabbit	Intravenous	50	27	17	4	
	Intramuscular	300	6	150	2	
	Oral	1,000	14	1,350	5	
	Peritoneal	—	—	50	4	
Chronic†						
Mouse	Hypodermic	300	4	300	4	6
Rat	Hypodermic	—	—	200	4	8
	Oral	350	13	500	9	5
Rabbit	Oral	250	7	—	—	19
Guinea-pig	Oral	1,000	6	1,000	6	4
Monkey	Oral	—	—	200	4	6

* LD 50 expressed as mg./kg.

† Maximum doses which permitted survival of all animals for the period stated. (Larger doses were fatal to some animals in a few hours).

REFERENCES.

Brown, G. L. (1938): J. Physiol., 92, 22P.
Elderfield, R. C. and Leonard, N. S. (1942): J. org. Chem., 7, p. 558.
Elderfield, R. C. and Hawkins, L. (1942): *Ibid.*, 7, p. 573.
Harvey, A. M. (1939): J. Physiol., 95, p. 45.
Sharp, T. M. (1934): J. chem. Soc., 287.
Sharp, T. M. (1938): *Ibid.*, 1353.
Hutchinson, M., Shaw, F. and Wragge, W. (1942): Austral. J. exp. Biol., 20, p. 69.
Sollman, T. (1936): Manual of Pharmacology, 5th Edition, p. 560.

SOME OBSERVATIONS ON THE EXCRETION OF KETO-STEROIDS AND OESTROGENS IN THE URINE OF SHEEP

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Sex hormones, both male and female, or their metabolic products, are found in the urine of both sexes of many species of animals (Marker, 1939; Smith, Smith and Pincus, 1937) and determinations of the amounts excreted give valuable information as to sexual activity.

In the human female the excretion of 17-keto-steroids gradually increases from 0·3 mg. per day at 3 years to 15 mg. per day at puberty (Gallagher, Peterson, Dorfman, Kenyon and Koch, 1937; Bodansky and Bodansky, 1940) and decreases slightly during pregnancy (Hain, 1938, 1939). Reports on a relationship of keto-steroid content of pregnancy urine to the sex of the foetus have been made, but the earlier investigations were conducted without first hydrolysing the urine (Goecke, 1936; Burrows, MacLeod and Warren, 1942).

It is of interest to note that both bulls' urine, the richest known source of pregnanediol (Marker, Wittle and Lawson, 1938), and mares' urine, which is rich in oestrogens, have little androgenic activity (Heard, 1938).

The urine of pregnant ewes has previously been considered devoid of 17-keto-steroids.

The oestrogens occur in the urine as oestradiol (Smith and Smith, 1939), oestriol and oestrone, the latter occurring free or conjugated with glycuronic acid (Cohen and Marrian, 1939). The ratio of free to combined oestrogens varies with the stage of pregnancy and rises abruptly just prior to parturition (Smith, Smith and Pincus, 1937).

The normal human female excretes about 250 I.U. of total oestrogens per day in the urine, and this excretion varies so that two peaks occur, one at 6–12 days before the onset of menstruation and one 6–15 days after. During pregnancy the excretion gradually rises to 10,000 I.U. per day in the urine (Hain, 1939) and considerable quantities are found in the faeces (Dohrn and Faure, 1939). Small amounts of oestrogenic substances have also been found in the urine of female castrates.

The urine of the mare is a very rich source of oestrogens which occur free or combined with glycuronic acid or even with sulphuric acid (Schachter and Marrian, 1938). There is an increase in excretion at oestrus and again 10–15 days after the onset (Zondek, 1934; Mayer, Andrews and McKenzie, 1940). During pregnancy there is a slight decrease from the peak at oestrus; the decreased values continue until the 30th day when an increase occurs, and this continues until a maximum is reached between 200 and 270 days (Cole and Saunders, 1935). No marked rise in free oestrogens has been detected in mares' urine just prior to parturition. If the ovaries are removed during pregnancy there is an immediate slight drop in excretion but the normal value is soon regained (Hart and Cole, 1935). Considerable amounts of oestrogens have been found in the faeces of pregnant mares. Three additional substances with slight oestrogenic activity, viz., hippulin, equilin and equilinin, have been isolated from pregnant mares' urine.

The pregnant cow excretes large quantities of oestrogens during the later stages of pregnancy with a possibility of an elevation in the third month (Hisaw and Mayer, 1929). These observations were made on unhydrolysed urine in which bacterial action may have liberated varying amounts of free oestrogens. According to Barry, Patterson and Underhill (1935), who give figures following acid hydrolysis, the excretion is low until the 30th week and rises suddenly to 17,000 mouse units per litre.

The sow's oestrogen excretion during pregnancy resembles that of the human. The goat has a maximum excretion in the second and third month. Reports for cats and deer are negative (Bomskov, 1939).

No oestrogens have hitherto been detected in the urine or blood of sheep of either sex (Hayston, 1939), but they have been recorded in the placenta, ovary and follicular fluid (Parkes and Bellerby, 1926).

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MATERIAL.

Preliminary investigations were made on pooled urine of two groups of eleven ewes in the early stages of pregnancy. The samples were collected by inhibiting the breathing of the animals. Only limited amounts of urine were collected and no figures for the total daily excretion could be obtained. One ewe was then selected from each group and placed in a metabolism cage. Every 12–16 days, samples were collected, the urine being received into 3 ml. of toluene and transferred daily to an ice-chest. After filtration the volume and specific gravity were determined and the filtrate used for the estimation of 17-keto-steroids and oestrogens.

METHODS.

The steroids were extracted by the method of Dingemanse, Borchart and Laquer (1937) as modified by Callow *et al.* (1939). A Hilger absorptiometer was used for the colorimetric assay of the 17-keto-steroids. The extracts have been retained for future bio-assay. The oestrogenic extracts were taken up in 9 ml. of arachis oil and this solution used in doses of 0·4 ml. in rat titration.

For the bio-assay of the oestrogenic extracts the methods of Burns (1937) and Emmens (1939) were used. Fifty-eight female rats were castrated at 5–6 weeks. After preliminary trials the rats were divided into 3 groups of 20, 19 and 19. For each test one group was kept as a control and injected with a known amount (0·1 I.U.) of oestrone in the same volume (0·4 ml.) of arachis oil as the test injections. Each group was used in turn as the control because castrates gradually became more sensitive to oestrogens and this increase in sensitivity is inhibited by the injection of oestrogenic substances. Thus the effect of the control injection is evenly distributed over the groups, and rats alternated in this manner may be used every 10 days for 12 months.

The rats were injected on the first day at 9 a.m. with 0·2 ml. of standard or unknown solution. This was repeated on the second day with the same dose and at the same time. Vaginal smears were taken on the third and fourth days at 9 a.m. and 4 p.m. by introducing a drop of water into the vagina with a clean pipette, allowing a few bubbles of air to be forced in to mix the contents, and withdrawing the drop which was smeared on the slide. The smear was dried, fixed with methyl alcohol and stained with polychrome methylene blue. Positive results consist of a smear composed mainly of keratinized or large epithelial cells and perhaps a few leucocytes. Incomplete reactions show evidence of epithelial cells with leucocytes in the minority. Negative results consist of leucocytes with perhaps a few epithelial cells.

The concentration of oestrogen was calculated from the standard curve by comparing the response produced by the unknown solution with that of the standard, which varies with each test, and expressing it as the equivalent of the pure substance. This was multiplied by 22·5 i.e. (9: 0·4) to give the total amount in the sample, and the daily excretion and the amount per litre were calculated.

Special precautions were taken to prevent keratinization produced from vitamin A deficiency or vaginal irritation that may be caused by taking smears too frequently (Coward, Combden and Lee, 1935).

Known amounts of oestrone and oestriol were added to some samples of which a control specimen was kept and the percentage recovery was calculated.

One rat exhibited persistent oestrus in tests 3 and 4 (see Tables) and when examined post-mortem a small body, showing characteristic luteal histology was found where the right ovary had apparently been removed.

RESULTS.

The results are given in detail in Tables 1 and 2. The urine of pregnant ewes carrying ram lambs was examined for 17-keto-steroids and oestrogens. 17-keto-steroids were found throughout pregnancy, while oestrogens were detected only during the last month with a probable increase prior to parturition; in this respect the excretion in the ewe resembles that of the cow.

By using the method described 50 p.c. of added oestriol was recovered from sheep's urine.

SUMMARY.

17-keto-steroids have been detected in the urine of two ewes carrying ram lambs.

The concentrations of keto-steroids in the urine of the two ewes examined were of the same order as those recorded for normal human females.

Oestrogens occurred in the urine of pregnant ewes during the last few weeks of pregnancy.

TABLE 1.

Assays of 17-keto-steroids and oestrogens in urine of sheep A.
Mated 9/6/41. Delivered 15-lb. still-born male lamb 3/11/41.

Sample.	Final collection date.	Period hours.	Volume ml.	Specific gravity.	Response p.c.	Oestrogen total I.U.	Oestrogen per litre I.U.	Oestrogen per day I.U.	Keto-steroid total mg.	Keto-steroid per litre mg.	Keto-steroid per day mg.	Remarks.
1	July 21		900	1,009	0				1·8	2·0		Urine collected from a group of eleven ewes in the same stage of pregnancy as sheep A.
2	Aug. 11		1,030	1,033	20	81	79		9·8	9·5		Same as sample 1. Oestrogen reactions incomplete.
3	Aug. 25	96	2,400	1,033	0				20·4	8·5	5·1	
4	Sept. 12	48	1,700	1,027	0				13·6	8·0	6·8	One rat exhibited persistent oestrus. Rejected.
5	Sept. 26	72	2,000	1,039					24·0	12·0	8·0	Flask broken, oestrogens not determined.
6a	Oct. 9	48	1,800	1,030	39	121	66	61	23·4	13·0	11·7	Reaction incomplete.
6b	Oct. 9	48	1,800	1,030	85	265			23·2	12·6	11·6	0·3 mg. oestriol added; 50 p.c. recovery.
7	Oct. 28	48	1,850	1,030	100	>400	>213	>200	39·2	21·2	19·6	Reactions completely positive.

TABLE 2.

Assays of 17-keto-steroids and oestrogens in urine of sheep B.
Mated 26/6/41. Delivered 12-lb. male lamb 16/11/41.

Sample.	Final collection date.	Period hours.	Volume ml.	Specific gravity.	Response p.c.	Oestrogen total I.U.	Oestrogen per litre I.U.	Oestrogen per day I.U.	Keto-steroid total mg.	Keto-steroid per litre mg.	Keto-steroid per day mg.	Remarks.
1	July 21		1,350	1,017					9·7	7·2		Urine collected from a group of eleven ewes in the same stage of pregnancy as sheep B.
2	Aug. 11	18†	2,400	1,027	6	30	29	40	12·0	5·0	16·0	One rat exhibited persistent oestrus. Rejected. One rat gave incomplete reaction. Receptacle overflowed.
3	Aug. 25	96	2,000	1,042	0				23·2	11·6	5·8	
4	Sept. 12	96	2,200	1,042	0				29·0	13·2	7·3	
5	Sept. 26	120	2,200	1,051	0				31·5	14·3	6·3	
6	Oct. 9	96	2,000	1,045	37	120	60	30	50·0	25·0	12·5	Residue of ether extract taken up directly in oil.
7	Oct. 27	72	1,700	1,041	100	>400	>236	>133	30·1	17·7	10·1	Oestrogen content above the range of the test group.

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REFERENCES.

Barrie, M. M. O., Patterson, J. B. E. and Underhill, S. W. F. (1935): J. Pharm. Pharmacol., 8, p. 424.
Bodansky, M. and Bodansky, O. (1940): "Biochemistry of Disease", Macmillan, New York.
Bomskov, C. (1939): "Methodik der Hormonforchung", Thieme, Leipzig.
Burn, J. H. (1937): "Biological Standardization", New York, London, Oxford, p. 130.
Burrows, H., MacLeod, D. H. and Warren, F. L. (1942): Nature, 149, p. 300.
Callow, N. H., Callow, R. K., Emmens, C. W. and Stroud, S. W. (1939): J. Endocrin., 1, p. 76.
Cohen, S. L. and Marrian, G. F. (1935): Biochem. J., 28, p. 1603.
Cole, H. H. and Saunders, L. (1935): Endocrinol., 19, p. 199.
Coward, K. H., Combden, M. R. and Lee, E. M. (1935): Biochem. J., 29, p. 2736.
Dingemanse, E., Borchart, H. and Laquer, E. (1937): *Ibid.*, 31, p. 500.
Dohrn, M. and Faure, W. (1928): Klin. Woch., 7, p. 943.
Emmens, C. W. (1939): Med. Res. Council Sp. Rep. Ser. 324, His Majesty's Stationery Office, London.
Gallagher, T. F., Peterson, D H., Dorfman, R. I., Kenyon, A. T. and Koch, F. C. (1937): J. clin. Invest., 16, p. 695.
Goecke, H. (1936): Z. Geburtchule und Gynakol., 112, p. 273.
Hain, A. M. (1938): Edin. med. J., 45, p. 678.
Hain, A. M. (1939): Quart. J. exp. Physiology, 29, p. 139.
Hart, G. H. and Cole, H. H. (1935): Amer. J. Physiol., 109, p. 320.
Heard, R. D. H. (1938): J. Amer. chem. Soc., 60, p. 493.
Hayston, J. T. (1939): Unpublished.
Hisaw, F. L. and Meyer, R. K. (1929): Proc. Soc. exp. Biol., 26, p. 586.
Marker, R. E., Lawson, E. J., Whittle, E. L. and Crooks, H. M. (1938): J. Amer. chem. Soc., 60, p. 1559.
Marker, R. E., Wittle, E. L. and Lawson, E. J (1938): *Ibid.*, 60, p. 2931.
Marker, R. E. (1939): *Ibid.*, 61, p. 944.
Mayer, D. T., Andrews, F. N. and McKenzie, F. F. (1940): Endocrinol., 27, p. 867.
Parkes, A. S. and Bellerby, C. W. (1926): J. Physiol., 61, p. 562.
Parkes, A. S. and Bellerby, C. W. (1926): *Ibid.*, 62, p. 383.
Schachter, B. and Marrian, G. F. (1938): J. biol. Chem., 126, p. 663.
Smith, G. V., Smith, O. W. and Pincus, G. (1937): Amer. J. Physiol., 121, p. 98.
Smith, O. W. and Smith, G. V. (1939): J. biol. Chem., 130, p. 431.
Zondek, B. (1934): Nature, 133, p. 494.